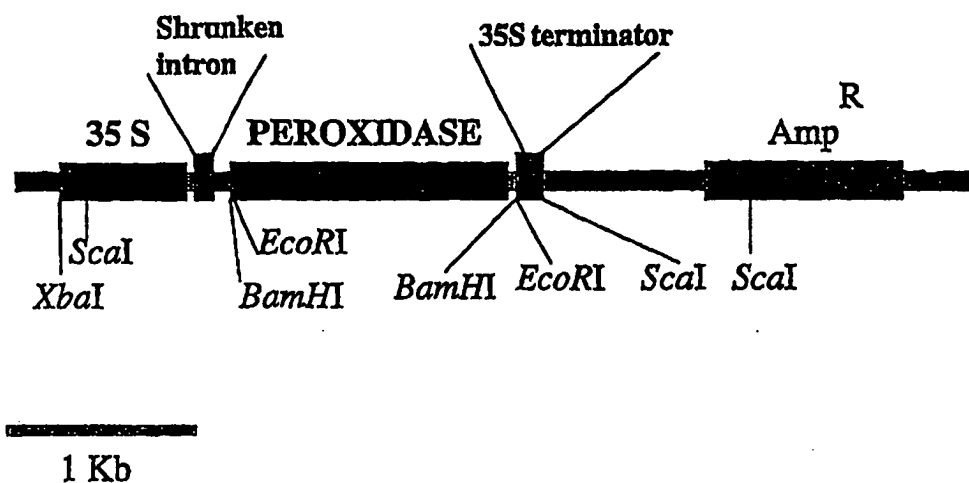




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**(54) Title:** METHODS FOR CONFERRING INSECT RESISTANCE TO A MONOCOT USING A PEROXIDASE CODING SEQUENCE

**(57) Abstract**

The present invention relates, in general, to methods and compositions for controlling insects in monocotyledonous plants (monocots), particularly maize. More precisely, the present invention relates to (1) a method for controlling insects comprising feeding or contacting an insect with an insecticidal amount of transgenic monocotyledonous plant cells comprising a recombinant DNA sequence comprising a coding sequence encoding peroxidase and (2) a fertile transgenic monocot plant comprising a recombinant DNA sequence comprising a coding sequence encoding peroxidase.

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**METHODS FOR CONFERRING INSECT RESISTANCE TO A MONOCOT**  
**USING A PEROXIDASE CODING SEQUENCE**

The present invention relates, in general, to methods and compositions for controlling insects in monocotyledonous plants (monocots), particularly maize. More precisely, the present invention relates to (1) a method for controlling insects comprising feeding or contacting an insect with an insecticidal amount of transgenic monocotyledonous plant cells comprising recombinant DNA comprising a coding sequence encoding peroxidase and (2) a fertile transgenic monocot plant comprising recombinant DNA comprising a coding sequence encoding peroxidase.

Insect pests are a major factor in the loss of the world's commercially important agricultural crops. Broad spectrum chemical pesticides have been used extensively to control or eradicate pests of agricultural importance. Although insecticides have been effective in controlling most harmful insects, there are considerable problems associated with the use of these compounds. Insecticides are expensive and costly to apply. Often repeated applications are necessary for effective control. There is also concern that insects have or will become resistant to many of the chemicals used in controlling them. Insecticides often kill beneficial insects which are pollinators or prey on the herbivorous insects. Additionally, there are environmental hazards associated with the long term use of chemical insecticides.

Programs of pest management are being introduced which lower the use of chemical insecticides. These programs include the improvement of crops by selection, the employment of biological control agents and insect predators, and the incorporation of insect resistant genes through breeding programs and genetic engineering. The most widely utilized genes for genetic engineering are the crystal protein genes from *Bacillus thuringiensis*. See, for example, Rice *et al.*, EP-A-292 435 and Koziel *et al.*, WO 93/07278. The majority of the crystal proteins made by *Bacillus* are toxic to larvae of insects in the orders Lepidoptera, Diptera and Coleoptera. In general, when an insecticidal crystal protein is ingested by a susceptible insect, the crystal is solubilized and acts as a toxic moiety. To avoid the development of insects which are resistant to these toxins, additional toxins are needed which have additive or synergistic affects.

Peroxidases are a subclass of oxido-reductases that use a peroxide such as  $H_2O_2$  as substrate. Peroxidases are heme-containing monomeric glycoproteins able to bind divalent cations (mainly  $Ca^{2+}$ , but also  $Mn^{2+}$ ) (Maranon and Van Huystee, *Phytochemistry* 37: 1217-1225 (1994)). The prosthetic groups for peroxidase have different roles. While the heme group is involved in catalysis, the divalent cations stabilize the heme moiety, and the glycosyl groups may help to stabilize the peroxidase by decreasing its turnover rate (Maranon and Van Huystee, *Phytochemistry* 37: 1217-1225 (1994)).

Peroxidases are often grouped into anionic, cationic, and neutral forms according to their migration on isoelectric focusing gels. Although as enzymes they are considered to have wide substrate specificity, they do appear to have some substrate "preferences" for different isoenzymes (Van Huystee, *Ann. Rev. Plant Physiol.*, 205-219 (1987)). There are several types of peroxidases and related enzymes including guaiacol peroxidase, NADH peroxidase, cytochrome-C peroxidase, catalase, glutathione peroxidase, L-ascorbate peroxidase, and manganese peroxidase.

In plants, peroxidases are monomeric proteins which are highly complex enzymes whose activities are closely regulated by the plant. Peroxidases are critical in the biosynthesis of plant cell walls. Peroxidases promote the peroxidative polymerization of the monolignols coniferyl, *p*-coumaryl, and sinapyl alcohol into lignin (Greisbach, *In: The Biochemistry of Plants*, Ed. Conn, Academic, New York pp. 457-480 (1991)). Different plant species have varying ratios of the monolignol species assembled in a semi-random fashion (Hwang *et al.*, *Carbohydrate Polymers* 14:77-88 (1991)). Lignification serves to strengthen and reinforce cell walls. The overall result is a toughening of the plant tissue.

A tobacco anionic peroxidase was utilized to transform *N. tabacum* and *N. sylvestris* (Lagrimini, *Plant Cell* 2:7-18 (1990); Lagrimini, *Plant Physiology* 96:577-583 (1991)). These transgenic plants constitutively overexpressed a tobacco anionic peroxidase from a 35S promoter. The most striking phenotype of peroxidase overexpression was chronic wilting which begins at approximately the time of flowering. In addition, the plants were retarded in growth, had smaller, compacted cells, and brown rapidly in response to wounding.

The same construct was also utilized to transform tomato plants (Lagrimini *et al.*, *J. Am. Soc. Hort. Sci.* 117:1012-1016 (1992); Lagrimini *et al.*, *Hortscience* 28:218-221 (1993)). These plants were also found to wilt severely after flowering, and showed excessive browning and reduced fruit size.

Initial studies have shown that some tissues of transgenic tobacco and tomato plants expressing a tobacco anionic peroxidase gene were resistant to some insects (Dowd *et al.*, presentation at the National Meeting of the Entomological Society of America, Indianapolis, December 1993). Tobacco and tomato are closely related dicots belonging to the same family, the Solanaceae. In contrast, the transgenic monocots of the present invention have vastly different physiology, biochemistry, anatomy, and metabolism when compared to dicots. For example, monocots have different codon usage, use C4 instead of C3 metabolism, have different fatty acid content, imperfect flowers, and the like. Thus, it was unknown whether substrates would exist in monocots that could be used by peroxidase to control insects.

Further, peroxidases are glycoproteins that must undergo specific post-transcriptional modification and incorporation of heme-containing groups to be stable and enzymatically active. Peroxidases are involved in the synthesis of secondary metabolites and lignins whose nature depends on the substrates available in the specific plant. Therefore, the final products obtained by expressing peroxidases may differ from plant to plant.

Additionally, resistance to corn earworms is negatively correlated to silk browning indicating that an increase in peroxidase would lower resistance (Byrne *et al.*, *Environ. Entomol.* 18:356-360 (1989)). This teaches away from using peroxidase to control insects in monocots.

Further, altered lignin production in corn (in *bm* mutants) causes increased susceptibility to insects (Barriere and Argillier, *Agronomie* 13:865-876 (1993)). Thus, a foreign peroxidase which alters lignification would not be expected to decrease susceptibility to insects. Further, it was unexpected from the teachings of Bergvinson *et al.*, *The Canadian Entomologist* 127: 111-122, 1995, that insect resistance is imparted to plants by toughening of tissues due to peroxidase activity in the early stages of growth.

Therefore, prior to the present invention, the effect of expressing a recombinant peroxidase in monocots was unpredictable.

Methods for controlling insects and insect resistant monocot plants are provided.

The invention, more specifically, provides a method for controlling insects comprising feeding or contacting an insect with an insecticidal amount of transgenic monocotyledonous plant cells comprising recombinant DNA comprising a coding sequence encoding

peroxidase, wherein expression of peroxidase confers insect resistance in the transgenic monocotyledonous plant cells.

The invention also provides a fertile transgenic monocotyledonous plant at least a part of which comprises cells with recombinant DNA comprising a coding sequence encoding peroxidase, wherein expression of peroxidase confers on the monocotyledonous plant a phenotypic trait.

The invention also provides a transgenic plant cell, tissue, or seed obtained from the above-described plant.

The invention further provides transgenic descendants of the above-described plant.

The invention also provides a transgenic plant cell, tissue, or seed obtained from the above-described descendants.

Further objects and advantages of the present invention will be clear from the description that follows.

FIGURE 1. The plasmid, pJS20293, is shown as containing tobacco anionic peroxidase inserted between (1) the CaMV 35S promoter linked to the shrunken intron and (2) the CaMV 35S terminator.

FIGURE 2. The plasmid, pUBIAc, is shown.

The following definitions will assist in the understanding of the present invention.

Plant cell: the structural and physiological unit of plants, consisting of a protoplast and the cell wall. The term "plant cell" refers to any cell which is either part of or derived from a plant. Some examples of cells include differentiated cells that are part of a living plant; differentiated cells in culture; undifferentiated cells in culture; the cells of undifferentiated tissue such as callus or tumors; differentiated cells of seeds, embryos, propagules and pollen.

Plant tissue: a group of plant cells organized into a structural and functional unit. Any tissue of a plant in planta or in culture is included. This term includes, but is not limited to, whole plants, plant organs, plant seeds, tissue culture and any groups of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

Protoplast: a plant cell without a cell wall.

Descendant plant: a sexually or asexually derived future generation plant which includes, but is not limited to, progeny plants.

Transgenic plant: a plant having stably incorporated recombinant DNA in its genome.

Recombinant DNA: Any DNA molecule formed by joining DNA segments from different sources and produced using recombinant DNA technology.

Recombinant DNA technology: Technology which produces recombinant DNA *in vitro* and transfers the recombinant DNA into cells where it can be expressed or propagated (See, Concise Dictionary of Biomedicine and Molecular Biology, Ed. Juo, CRC Press, Boca Raton (1996)), for example, transfer of DNA into a protoplast(s) or cell(s) in various forms, including, for example, (1) naked DNA in circular, linear or supercoiled forms, (2) DNA contained in nucleosomes or chromosomes or nuclei or parts thereof, (3) DNA complexed or associated with other molecules, (4) DNA enclosed in liposomes, spheroplasts, cells or protoplasts or (5) DNA transferred from organisms other than the host organism (ex. *Agrobacterium tumefaciens*). These and other various methods of introducing the recombinant DNA into cells are known in the art and can be used to produce the transgenic cells or transgenic plants of the present invention.

Recombinant DNA technology also includes the homologous recombination methods described in Treco *et al.*, WO 94/12650 and Treco *et al.*, WO 95/31560 which can be applied to increasing peroxidase activity in a monocot. Specifically, regulatory regions (ex. promoters) can be introduced into the plant genome to increase the expression of the endogenous peroxidase.

Also included as recombinant DNA technology is the insertion of a peroxidase coding sequence lacking selected expression signals into a monocot and assaying the transgenic monocot plant for increased expression of peroxidase due to endogenous control sequences in the monocot. This would result in an increase in copy number of peroxidase coding sequences within the plant.

The initial insertion of the recombinant DNA into the genome of the  $R^0$  plant is not defined as being accomplished by traditional plant breeding methods but rather by technical methods as described herein. Following the initial insertion, transgenic descendants can be propagated using essentially traditional breeding methods.

Chimeric gene: A DNA molecule containing at least two heterologous parts, e.g., parts derived from pre-existing DNA sequences which are not associated in their

pre-existing states, these sequences having been preferably generated using recombinant DNA technology.

Expression cassette: a DNA molecule comprising a promoter and a terminator between which a coding sequence can be inserted.

Coding sequence: a DNA molecule which, when transcribed and translated, results in the formation of a polypeptide or protein.

Gene: a discrete chromosomal region comprising a regulatory DNA sequence responsible for the control of expression, i.e. transcription and translation, and of a coding sequence which is transcribed and translated to give a distinct polypeptide or protein.

Phenotypic trait: an observable property resulting from the expression of one or more genes.

The present invention relates to methods for controlling insects such as Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, and Trichoptera. Particular examples of such insect pests are European corn borer, stalk corn borer, black cutworm, corn earworm, fall armyworm, southwestern corn borer, lesser cornstalk borer, sugarcane borer, western corn rootworm, northern corn rootworm, southern corn rootworm, wireworms, northern masked chafer, southern masked chafer, Japanese beetle, corn flea beetle, maize billbug, corn leaf aphid, corn root aphid, chinch bug, redlegged grasshopper, migratory grasshopper, seedcorn maggot, corn blotch leafminer, grass thrips, thief ant and two-spotted spider mite. Any method wherein expression of peroxidase confers insect resistance in monocotyledonous plant cells is encompassed by the present invention.

In one preferred embodiment, the present invention relates to a method for controlling insects by feeding or contacting an insect with an insecticidal amount of transgenic monocotyledonous plant cells, wherein the genome of said plant cells encodes an enzyme with peroxidase activity. Upon expression the peroxidase confers insect resistance to the transgenic plant cells. The transgene encoding the peroxidase constitutes an additional gene inserted into the genome of a progenitor plant which does not naturally encode said peroxidase.

In a further preferred embodiment, the homologous recombination methods described in Treco *et al.*, WO 94/12650 and Treco *et al.*, WO 95/31560 are used to

increase peroxidase activity in a monocot and thus, insect resistance. Specifically, regulatory regions (ex. promoters) are introduced into the plant genome to increase the expression of the endogenous peroxidase which increases the insect resistance of the plant.

In another preferred embodiment, the present invention relates to the insertion of a peroxidase coding sequence lacking selected expression signals into a monocot and assaying the transgenic monocot plant for increased expression of peroxidase due to endogenous control sequences in the monocot. This results in an increase in copy number of peroxidase coding sequences within the plant.

In a further preferred embodiment, the present invention relates to a method of increasing the copy number of the endogenous peroxidase gene wherein insect resistance is conferred on the monocot plant. Such a method is preferably accomplished using traditional plant breeding methods or using tissue culture techniques.

Insect resistant plants comprise increased insect resistance over that found in native non-manipulated plants due to increased levels of peroxidase.

The invention further relates to a commercial bag comprising seed of a monocotyledonous plant transformed with recombinant DNA comprising a coding sequence encoding peroxidase, wherein expression of peroxidase confers on said plant a phenotypic trait. Preferred within this invention is a commercial bag comprising seed of a transgenic plant wherein expression of peroxidase confers on said plant insect resistance or standability. A further preferred object of the invention is such a commercial bag together with label instructions for the use of the seed contained therein.

#### *Insect Resistance*

Preferably, the transgenic monocots of the present invention are resistant to insects selected from the orders including but not limited to Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, particularly, Coleoptera and Lepidoptera. For purposes of the present invention, it is recognized that the transgenic plants of the invention can be resistant to not only insects but also fungi, bacteria, nematodes, mites, and the like.

Maize plants of the present invention are preferably resistant to an insect or insects selected from the group which includes but is not limited to *Ostrinia nubilalis*, European corn

borer; *Sesemia nonagrioides*, stalk corn borer; *Agrotis ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, sugarcane borer; *Diabrotica virgifera virgifera*, western corn rootworm, *Diabrotica longicornis barberi*, northern corn rootworm, *Diabrotica undecimpunctata howardi*, southern corn rootworm, *Melanotus* spp., wireworms, *Cyclocephala borealis*, northern masked chafer (white grub), *Cyclocephala immaculata*, southern masked chafer (white grub), *Popillia japonica*, Japanese beetle (grub and adult forms), *Chaetocnema pulicaria*, corn flea beetle, *Sphenophorus maidis*, maize billbug, *Rhopalosiphum maidis*, corn leaf aphid, *Anuraphis maidiradicis*, corn root aphid, *Blissus leucopterus leucopterus*, chinch bug, *Melanoplus femurrubrum*, redlegged grasshopper, *Melanoplus sanguinipes*, migratory grasshopper, *Hylemya platura*, seedcorn maggot, *Agromyza parvicornis*, corn blotch leafminer, *Anaphothrips obscurus*, grass thrips, *Solenopsis milesta*, thief ant, and *Tetranychus urticae*, two-spotted spider mite.

Sorghum plants of the present invention are preferably resistant to an insect or insects selected from the group which includes but is not limited to *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranean*, granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes*, *Conoderus*, and *Aeolus* spp., wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Sipha flara*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; and *Tetranychus urticae*, twospotted spider mite.

Wheat plants of the present invention are preferably resistant to an insect or insects selected from the group which includes but is not limited to *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, pale western cutworm; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza*

*americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella fusca*, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; and *Aceria tulipae*, wheat curl mite.

Rice plants of the present invention are preferably resistant to an insect or insects selected from the group which includes but is not limited to *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; and *Acrosternum hilare*, green stink bug.

Barley plants of the present invention are preferably resistant to an insect or insects selected from the group which includes but is not limited to *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servos*, brown stink bug; *Hylemya platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Thysanoptera*, Thrips; and *Petrobia latens*, brown wheat mite.

In one embodiment, the present invention relates to a fertile transgenic monocotyledonous plant (monocot) comprising recombinant DNA comprising a coding sequence encoding peroxidase.

Monocots are plants whose embryo has one cotyledon. Monocots are one of the two large classes of angiosperms (dicots being the other large class).

Preferred families within the monocot class include: gramineae (grass family; preferred members of the gramineae include forage grasses (ex. *Festuca* (fescue grass)), *Hordeum* (barley), *Avena* (oat), *Zea mays* (corn), *Triticum* (wheat), *Secale* (rye), *Sorghum vulgare* (sorghum), and *Oryza sativa* (rice)); liliaceae (lily family; preferably *Allium* (onion) and *Asparagus*); and Dioscoreaceae (yam family) all of which are encompassed by the present invention. The present invention also includes but is not limited to monocot species, for example, preferred lines of *Zea mays* include Funk 5N984, Funk 5N986, Funk 2717, Funk 211D, Funk 2N217A, B73, A632, CM105, B37, B84, B14, Mo17, A188, CG00526, CG00615 and CG00714.

The genetic properties engineered into the transgenic seeds and plants mentioned above are passed on by sexual reproduction or vegetative growth and can thus be maintained and propagated in descendant plants. Generally said maintenance and

propagation make use of known agricultural methods developed to fit specific purposes such as tilling, sowing or harvesting. Specialized processes such as hydroponics or greenhouse technologies can also be applied. As the growing crop is vulnerable to attack and damages caused by insects or infections as well as to competition by weed plants, measures are undertaken to control weeds, plant diseases, insects, nematodes, and other adverse conditions to improve yield. These include mechanical measures such a tillage of the soil or removal of weeds and infected plants, as well as the application of agrochemicals such as herbicides, fungicides, gametocides, nematocides, growth regulants, ripening agents and insecticides.

Use of the advantageous genetic properties of the transgenic plants and seeds according to the invention can further be made in plant breeding which aims at the development of plants with improved properties such as tolerance of pests, herbicides, or stress, improved nutritional value, increased yield, or improved structure causing less loss from lodging or shattering. The various breeding steps are characterized by well-defined human intervention such as selecting the lines to be crossed, directing pollination of the parental lines, or selecting appropriate descendant plants. Depending on the desired properties different breeding measures are taken. The relevant techniques are well known in the art and include but are not limited to hybridization, inbreeding, backcross breeding, multiline breeding, variety blend, interspecific hybridization, aneuploid techniques, etc. Hybridization techniques also include the sterilization of plants to yield male or female sterile plants by mechanical, chemical or biochemical means. Cross pollination of a male sterile plant with pollen of a different line assures that the genome of the male sterile but female fertile plant will uniformly obtain properties of both parental lines. Thus, the transgenic seeds and plants according to the invention can be used for the breeding of improved plant lines which for example increase the effectiveness of conventional methods such as herbicide or pestidice treatment or allow to dispense with said methods due to their modified genetic properties. Alternatively new crops with improved stress tolerance can be obtained which, due to their optimized genetic "equipment", yield harvested product of better quality than products which were not able to tolerate comparable adverse developmental conditions.

In seeds production germination quality and uniformity of seeds are essential product characteristics, whereas germination quality and uniformity of seeds harvested and sold by the farmer is not important. As it is difficult to keep a crop free from other crop and

weed seeds, to control seedborne diseases, and to produce seed with good germination, fairly extensive and well-defined seed production practices have been developed by seed producers, who are experienced in the art of growing, conditioning and marketing of pure seed. Thus, it is common practice for the farmer to buy certified seed meeting specific quality standards instead of using seed harvested from his own crop. Propagation material to be used as seeds is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bactericides, nematocides, molluscicides or mixtures thereof. Customarily used protectant coatings comprise compounds such as captan, carboxin, thiram (TMTD<sup>®</sup>), methalaxyl (Apron<sup>®</sup>), and pirimiphos-methyl (Actellic<sup>®</sup>). If desired these compounds are formulated together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal or animal pests. The protectant coatings may be applied by impregnating propagation material with a liquid formulation or by coating with a combined wet or dry formulation. Other methods of application are also possible such as treatment directed at the buds or the fruit.

It is a further aspect of the present invention to provide new agricultural methods such as the methods exemplified above which are characterized by the use of transgenic plants, transgenic plant material, or transgenic seed according to the present invention.

In another embodiment, the present invention relates to a transgenic plant cell, tissue, organ, seed or plant part obtained from the transgenic plant. Also included within the invention are transgenic descendants of the plant as well as transgenic plant cells, tissues, organs, seeds and plant parts obtained from the descendants.

As described herein, the present invention relates to a fertile transgenic monocot plant transformed with a peroxidase coding sequence. Preferably, the peroxidase coding sequence confers on the monocot plant a phenotypic trait that is not found in a parent plant lacking the peroxidase coding sequence or its overexpression. Phenotypic traits which can be produced include insect resistance and enhanced standability.

More preferably, the peroxidase coding sequence in the transgenic plant is sexually transmitted. In one preferred embodiment, the peroxidase coding sequence is sexually transmitted through a complete normal sexual cycle of the R0 plant to the R1 generation. Additionally preferred, the peroxidase coding sequence is expressed so that the level of

peroxidase in the cells, tissues, seeds or plant is increased above the level in the cells, tissues, seeds or plant of a monocot which only differ in that the peroxidase coding sequence is absent.

In one preferred embodiment, the peroxidase coding sequence is an anionic, cationic, or neutral peroxidase coding sequence. In another preferred embodiment, the peroxidase is a guaiacol peroxidase, NADH peroxidase, cytochrome-C peroxidase, catalase, glutathione peroxidase, L-ascorbate peroxidase, manganese peroxidase, hydrogen peroxide generating peroxidase, and/or lignin forming peroxidase.

A variety of peroxidase coding sequences are available in the art and are available for use in the present invention. For example, peroxidases have been cloned from tobacco (Lagrimini, M., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7542-7546 (1987)), potato (Roberts *et al.*, *Plant Molecular Biology* 11:5-26 (1988)), horseradish (Fujiyama *et al.*, *European Journal of Biochemistry*, 173, 681-687 (1988); Fujiyama *et al.*, *Gene* 89:163-169 (1990); and Welinder, K.G., *European Journal of Biochemistry* 96:483-502 (1979)), tomato (Roberts, E. and Kolattukudy, P.E., *Molecular Genes and Genetics*, 217, 223-232 (1989)), peanut (Buffard *et al.*, *Proc. Natl. Acad. Sci. USA* 87:8874-8878 (1990)), cucumber (Morgens *et al.*, *Plant Molecular Biology* 14:715-725 (1990)), *Arabidopsis* (Intapruk *et al.*, *Gene* 98:237-241 (1991)), wheat (Hertig *et al.*, *Plant Molecular Biology* 16:171-174 (1991); and Rebmann *et al.*, *Plant Molecular Biology* 16:329-331 (1991)), barley (Rasmussen *et al.*, *Plant Molecular Biology* 16:317-327 (1991); and Theilade, B. and Rasmussen, S.K., *Gene* 118:261-266 (1992)), rice (Reimman *et al.*, *Plant Physiology* 100:1611-1612 (1992)), maize (Hwang, Ph.D. thesis, Ohio State University), and turnip (Mazza and Welinder, *European Journal of Biochemistry* 108:481-489 (1980)).

The peroxidase coding sequences used in the present invention are not to be limited to known peroxidase coding sequences. Novel peroxidase coding sequences for use in the invention can be isolated by identity or similarity with known sequences. The tobacco anionic peroxidase shows identity or similarity with the amino acid sequences of the horseradish cationic peroxidase and the turnip cationic peroxidase. The overall identity or similarity between tobacco and horseradish is 52%; for tobacco and turnip, the peroxidases have 46% identity or similarity. Furthermore, there are regions in the peroxidase coding sequence where the identity or similarity approaches 100%. Four of these conserved regions correspond to domains critical for general peroxidase activity. Accordingly, DNA sequences from the conserved regions can be utilized to generally clone peroxidase coding

sequences from any plant species using methods well known in the art (*See, for example, Current Protocols in Molecular Biology, eds.: Ausubel et al., John Wiley & Sons, Inc., New York, NY (Spring 1996)*).

Likewise, novel peroxidase coding sequences can be isolated using antibodies made against one peroxidase enzyme to isolate other peroxidase enzymes. Homology between the various peroxidase isozymes has been demonstrated with antibodies made to the tobacco anionic peroxidase. By immunoblot analysis these antibodies cross-reacted strongly with the horseradish and turnip isozymes and also cross-reacted with most of the other tobacco isozymes. *See, Lagrimini, M., et al., Proc. Natl. Acad. Sci. USA 84:7542-7546 (1987)*. The novel peroxidase enzymes can be sequenced using methods well known in the art and their corresponding coding sequences can be isolated using methods well known in the art (For example see, Sambrook *et al.* *Molecular Cloning-A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, NY, USA (1988)).

The transgenic expression in plants of peroxidase coding sequences derived from sources other than the host plant (for example, from bacterial sources) can require the modification of those coding sequences to achieve and optimize their expression in the host plant. In some cases, modification to the coding sequences and adjacent sequence will not be required. It is sufficient to isolate a fragment containing the coding sequence of interest and to insert it downstream of a plant promoter. For example see, Gaffney *et al., Science* 261: 754-756 (1993). Preferably, as little adjacent microbial sequence should be left attached upstream of the ATG and downstream of the stop codon.

The peroxidase coding sequence can be optimized for enhanced expression in the host monocot. For example, since the preferred codon usage and codon frequency in the host plant can differ from the usage and frequency of the peroxidase coding sequence of interest, comparison of the usage and frequency of codons within a cloned coding sequence to the usage and frequency in plant coding sequences (and in particular coding sequences from the target plant) enables an identification of the codons within the coding sequence which can preferably be changed. The preferred codons can be determined from the codons of highest frequency in the proteins expressed in the largest amount in the plant or from the codons most preferred in the plant. *See, for example, Adang et al., EP-A-359 472; Fischhoff et al., EP-A-385 962; Cornelissen et al., WO 91/16432; Koziel et al., WO 93/07278; Perlak et al., Proc. Natl. Acad. Sci. USA 88:3324-3328 (1991); and Murray et al., Nucleic Acids Research 17:477-498 (1989).*

In this manner, the nucleotide sequences can be optimized for expression in the specific plant of interest. It is recognized that all or any part of the coding sequence can be optimized or synthetic. That is, synthetic or partially optimized sequences can also be used.

Plants differ from microorganisms in that their messages do not possess a defined ribosome binding site. Rather, ribosomes attach to the 5' end of the message and scan for the first available ATG at which to start translation. In plants, there is a preference for certain nucleotides adjacent to the ATG and thus, expression of microbial genes can be enhanced by the inclusion of a eukaryotic consensus translation initiator at the ATG. Clontech (1993/1994 catalog, page 210) have suggested a sequence as a consensus translation initiator for the expression of the *E. coli uidA* gene in plants. Further, Joshi, *Nucl. Acid Res.* 15: 6643-6653 (1987) has compared many plant sequences adjacent to the ATG and also suggests a consensus sequence. In situations where difficulties are encountered in the expression of microbial coding sequences in plants, inclusion of one of these sequences at the initiating ATG is preferred.

Coding sequences cloned from non-plant sources can also contain motifs which can be recognized in plants as 5' or 3' splice sites, thus generating truncated or deleted messages. These sites can be removed using the techniques known in the art (*See, for example, Current Protocols in Molecular Biology, eds.: Ausubel et al., John Wiley & Sons, Inc., New York, NY (Spring 1996)*).

A recombinant DNA comprising a coding sequence encoding a peroxidase can be used for producing transgenic plant tissues. A plant is preferably transformed with at least one recombinant DNA which can further comprise a transcriptional initiation region and a promoter both of which are operably linked to the peroxidase coding sequence.

The transcriptional initiation regions can be native or foreign to the host. By foreign it is intended that the transcriptional initiation region is not found in the wild type host into which the transcriptional initiation region is introduced.

The termination region can be obtained from (1) the same gene from which the transcriptional initiation region was obtained, (2) the peroxidase gene used or (3) derived from other source.

The peroxidase coding sequence is preferably operably fused to a plant expressible promoter, preferable promoters include constitutive, inducible, temporally regulated, developmentally regulated, chemically regulated, tissue-preferred and/or tissue-specific promoters. In one preferred embodiment, the peroxidase coding sequence is operably

linked to its naturally occurring promoter and/or polyadenylation signal sequence.

Preferred constitutive promoters include the CaMV 35S and 19S promoters (Fraley *et al.*, U.S. Patent No. 5,352,605). An additionally preferred promoter is derived from any one of several of the actin genes, which are known to be expressed in most cell types. The promoter expression cassettes described by McElroy *et al.*, *Mol. Gen. Genet.* 231: 150-160 (1991) can be easily modified for the expression of the peroxidase coding sequence and are particularly suitable for use in monocotyledonous hosts.

Yet another preferred constitutive promoter is derived from ubiquitin, which is another gene product known to accumulate in many cell types. The ubiquitin promoter has been cloned from several species for use in transgenic plants (*e.g.* sunflower - Binet *et al.* *Plant Science* 79: 87-94 (1991), maize - Christensen *et al.* *Plant Molec. Biol.* 12: 619-632 (1989)). The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence and vectors constructed for monocot transformation are disclosed in Christiansen *et al.*, EP-A-342 926. The ubiquitin promoter is suitable for the expression of the peroxidase coding sequence in transgenic plants, especially monocotyledons.

Tissue-specific or tissue-preferential promoters useful for the expression of the peroxidase coding sequence in plants, particularly maize, are those which direct expression in root, pith, leaf or pollen. Such promoters are disclosed in Koziel *et al.*, WO 93/07278. Chemically inducible promoters useful for directing the expression of the peroxidase coding sequence in plants are also preferred (See, Alexander *et al.*, WO 95/19443).

In addition to promoters, a variety of transcriptional terminators are also available for use in chimeric gene construction using a peroxidase coding sequence. Transcriptional terminators are responsible for the termination of transcription beyond the transgene and its correct polyadenylation. Appropriate transcriptional terminators and those which are known to function in plants include the CaMV 35S terminator, the *tm1* terminator, the pea *rbcS* E9 terminator and others known in the art. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Rosenberg *et al.*, *Gene*, 56:125 (1987); Guerineau *et al.*, *Mol. Gen. Genet.*, 262:141-144 (1991); Proudfoot, *Cell*, 64:671-674 (1991); Sanfacon *et al.*, *Genes Dev.*, 5:141-149; Mogen *et al.*, *Plant Cell*, 2:1261-1272 (1990); Munroe *et al.*, *Gene*, 91:151-158 (1990); Ballas *et al.*, *Nucleic Acids Res.* 17:7891-7903 (1989); Joshi *et al.*, *Nucleic Acid Res.*, 15:9627-9639 (1987)).

Numerous sequences have also been found to enhance gene expression from

within the transcriptional unit and these sequences can be used in conjunction with the peroxidase coding sequence to increase expression in transgenic plants. Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *Adh1* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells (Callis *et al.*, *Genes Devel.* 1: 1183-1200 (1987)). Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

The construct can also include a regulator such as a nuclear localization signal (Kalderon *et al.* *Cell* 39:499-509 (1984); and Lassner *et al.*, *Plant Molecular Biology* 17:229-234 (1991)), plant translational consensus sequence (Joshi, C.P., *Nucleic Acids Research* 15:6643-6653 (1987)), an intron (Luehrsen and Walbot, *Mol. Gen. Genet.* 225:81-93 (1991)), and the like, operably linked to the appropriate nucleotide sequence.

Preferably, the 5' leader sequence is included in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T.R., and Moss, *Proc. Natl. Acad. Sci. USA* 86:6126-6130 (1989)); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison *et al.*, MDMV leader (Maize Dwarf Mosaic Virus); *Virology*, 154:9-20 (1986)), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak, D.G., and Samow, P., *Nature* 353:90-94 (1991); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling, S.A., and Gebrke, L., *Nature*, 325:622-625 (1987)); tobacco mosaic virus leader (TMV) (Gallie, D.R. *et al.*, *Molecular-Biology of RNA*, pages 237-256 (1989)); and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. *et al.*, *Virology* 91:382-385 (1991)). See also, Della-Cioppa *et al.*, *Plant Physiology* 84:965-968 (1987).

In preparing the recombinant DNA, various DNA fragments can be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Towards this end, adapters or linkers can be employed to join the DNA fragments or other manipulations can be used to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resection, ligation, or the like is preferably employed, where insertions, deletions or substitutions, e.g. transitions and transversions, are involved.

Numerous transformation vectors are available for plant transformation, and the peroxidase coding sequences can be used in conjunction with any such vectors. The selection of a vector for use will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers are preferred. Selection markers used routinely in transformation include the *nptII* gene which confers resistance to kanamycin and related antibiotics (Messing & Vierra, *Gene* 19: 259-268 (1982); Bevan *et al.*, *Nature* 304:184-187 (1983)), the *bar* gene which confers resistance to the herbicide phosphinothricin (White *et al.*, *Nucl. Acids Res.* 18: 1062 (1990), Spencer *et al.*, *Theor. Appl. Genet.* 79: 625-631 (1990)), the *hph* gene which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, *Mol. Cell. Biol.* 4: 2929-2931), and the *dhfr* gene, which confers resistance to methotrexate (Bourouis *et al.*, *EMBO J.* 2: 1099-1104 (1983)).

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, *Nucl. Acids Res.* 12(22): 8711-8721 (1984)). In one preferred embodiment, the peroxidase coding sequence can be inserted into either of the binary vectors pCIB200 and pCIB2001 for use with *Agrobacterium*. These vector cassettes for *Agrobacterium*-mediated transformation can be constructed in the following manner. pTJS75kan was created by *NarI* digestion of pTJS75 (Schmidhauser & Helinski, *J Bacteriol.* 164: 446-455 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII (Messing & Vierra, *Gene* 19: 259-268 (1982); Bevan *et al.*, *Nature* 304: 184-187 (1983); McBride *et al.*, *Plant Molecular Biology* 14: 266-276 (1990)). *XhoI* linkers were ligated to the *EcoRV* fragment of pCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein *et al.*, *Gene* 53: 153-161 (1987)), and the *XhoI*-digested fragment was cloned into *SalI*-digested pTJS75kan to create pCIB200 (see also EP-A-332 104, example 19). pCIB200 contains the following unique polylinker restriction sites: *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, and *SalI*. pCIB2001 is a derivative of pCIB200 which created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI*, *SstI*, *KpnI*, *BglII*, *XbaI*, *SalI*, *MluI*, *BclI*, *AvrII*, *ApaI*, *HpaI*, and *StuI*. pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization

between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

An additional vector useful for *Agrobacterium*-mediated transformation is the binary vector pCIB10 which contains a gene encoding kanamycin resistance for selection in plants, T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium*. Its construction is described by Rothstein *et al.*, *Gene* 53: 153-161 (1987). Various derivatives of pCIB10 have been constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz *et al.*, *Gene* 25: 179-188 (1983). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717).

One such vector useful for direct gene transfer techniques in combination with selection by the herbicide Basta (or phosphinothricin) is pCIB3064. This vector is based on the plasmid pCIB246, which comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in Koziel *et al.*, WO 93/07278. The gene providing resistance to phosphinothricin is the *bar* gene from *Streptomyces hygroscopicus* (Thompson *et al.*, *EMBO J.* 6: 2519-2523 (1987)). This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

An additional transformation vector is pSOG35 which utilizes the *E. coli* gene dihydrofolate reductase (DHFR) as a selectable marker conferring resistance to methotrexate. PCR was used to amplify the 35S promoter (~800 bp), intron 6 from the maize *Adh1* gene (~550 bp; see Dennis *et al.*, *Nucleic Acid Res.* 12:3983-4000 (1984)) and 18 bp of the GUS untranslated leader sequence (see Jefferson *et al.*, *Proc. Nat. Acad. Sci. USA* 83: 8447-8451 (1986)). A 250 bp fragment encoding the *E. coli* dihydrofolate reductase type II gene was also amplified by PCR and these two PCR fragments were assembled with a *SacI-PstI* fragment from pBI221 (Clontech) which comprised the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generated pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generated the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and

have *HindIII*, *SphI*, *PstI* and *EcoRI* sites available for the cloning of foreign sequences.

The recombinant DNA described above can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway et al., *BioTechniques* 4:320-334 (1986)), electroporation (Riggs et al., *Proc. Natl. Acad. Sci. USA* 83:5602-5606 (1986), *Agrobacterium*-mediated transformation (Hinchee et al., *Biotechnology* 6:915-921 (1988); See also, Ishida et al., *Nature Biotechnology* 14:745-750 (June 1996) for maize transformation), direct gene transfer (Paszkowski et al., *EMBO J.* 3:2717-2722 (1984); Hayashimoto et al., *Plant Physiol.* 93:857-863 (1990)(rice)), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (see, for example, Sanford et al., U.S. Patent 4,945,050; and McCabe et al., *Biotechnology* 6:923-926 (1988)). See also, Weissinger et al., *Annual Rev. Genet.* 22:421-477 (1988); Sanford et al., *Particulate Science and Technology* 5:27-37 (1987)(onion); Svab et al., *Proc. Natl. Acad. Sci. USA* 87: 8526-8530 (1990) (tobacco chloroplast); Christou et al., *Plant Physiol.* 87:671-674 (1988)(soybean); McCabe et al., *Bio/Technology* 6:923-926 (1988)(soybean); Klein et al., *Proc. Natl. Acad. Sci. USA*, 85:4305-4309 (1988)(maize); Klein et al., *Bio/Technology* 6:559-563 (1988) (maize); Klein et al., *Plant Physiol.* 91:440-444 (1988) (maize); Fromm et al., *Bio/Technology* 8:833-839 (1990); and Gordon-Kamm et al., *Plant Cell* 2: 603-618 (1990) (maize); Koziel et al., *Biotechnology* 11: 194-200 (1993)(maize); Shimamoto et al., *Nature* 338: 274-277 (1989) (rice); Christou et al., *Biotechnology* 9: 957-962 (1991) (rice); Datta et al., *Bio/Technology* 8:736-740 (1990)(rice); European Patent Application EP-A-332 581 (orchardgrass and other *Pooideae*); Vasil et al., *Biotechnology* 11: 1553-1558 (1993) (wheat); Weeks et al., *Plant Physiol.* 102: 1077-1084 (1993) (wheat); Wan et al., *Plant Physiol.* 104: 37-48 (1994)(barley); Jahne et al., *Theor. Appl. Genet.* 89:525-533 (1994)(barley); Umbeck et al., *Bio/Technology* 5: 263-266 (1987)(cotton); Casas et al., *Proc. Natl. Acad. Sci. USA* 90:11212-11216 (Dec. 1993)(sorghum); Somers et al., *Bio/Technology* 10:1589-1594 (Dec. 1992)(oat); Torbert et al., *Plant Cell Reports* 14:635-640 (1995)(oat); Weeks et al., *Plant Physiol.* 102:1077-1084 (1993)(wheat); Chang et al., WO 94/13822 (wheat) and Nehra et al., *The Plant Journal* 5:285-297 (1994)(wheat).

One particularly preferred set of embodiments for the introduction of recombinant

DNA molecules into maize by microprojectile bombardment can be found in Koziel *et al.*, *Biotechnology* 11: 194-200 (1993), Hill *et al.*, *Euphytica* 85:119-123 (1995) and Koziel *et al.*, *Annals of the New York Academy of Sciences* 792:164-171 (1996). An additional preferred embodiment is the protoplast transformation method for maize as disclosed in Shillito *et al.*, EP-A-292 435.

Transformation of plants can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with the peroxidase coding sequence.

Methods using either a form of direct gene transfer, particle gun technology or *Agrobacterium*-mediated transfer usually, but not necessarily, are undertaken with a selectable or screenable marker which provide resistance to an antibiotic (e.g., kanamycin, hygromycin or methotrexate) or a herbicide (e.g., phosphinothricin). Examples of such markers are neomycin phosphotransferase, hygromycin phosphotransferase, dihydrofolate reductase, phosphinothricin acetyltransferase, 2, 2-dichloropropionic acid dehalogenase, acetohydroxyacid synthase, 5-enolpyruvyl-shikimate-phosphate synthase, haloarylnitrilase, acetyl-coenzyme A carboxylase, dihydropteroate synthase, chloramphenicol acetyl transferase, and  $\beta$ -glucuronidase. The choice of selectable or screenable marker for plant transformation is not, however, critical to the invention.

The peroxidase coding sequence is preferably used alone or in combination. That is, one or more peroxidase coding sequence can be inserted into a plant to control different insect pests. This can be accomplished by (1) transforming a host plant with a DNA sequence comprising more than one peroxidase coding sequence, (2) transforming a host plant with a DNA sequence comprising a single peroxidase coding sequence and identifying multiple insertions of the DNA sequence into the host genome, or (3) repeated transformation of a host plant with a peroxidase coding sequence until the host plant comprises the desired number of peroxidase coding sequences.

The level of insect protection of a plant against a given insect and/or its spectrum of insecticidal activities can also be increased by combining a peroxidase coding sequence with other coding sequences encoding proteins capable of controlling insects.

*Bacillus thuringiensis* (Bt) is a gram-positive, spore-forming bacterium which produces a parasporal crystal during sporulation (For review see, Koziel *et al.*, *Biotech. and Gen. Engin. Reviews* 11:171-228 (1993)). These crystals are predominantly comprised of

one or more proteins, called  $\delta$ -endotoxins or insecticidal crystal proteins, known to possess insecticidal activity when ingested by certain insects. Numerous strains of Bt are currently known. Each strain produces differing numbers of  $\delta$ -endotoxins with various insecticidal activities. Examples of Bt endotoxins which can be used in combination with peroxidases include, but are not restricted to CryIA(b) (Koziel *et al.*, *Bio/Technology* 11: 194-200 (1993)), CryIA(c) (US Patent 5,530,197), Cry1H (also called *Cry9C*) (Lambert *et al. Appl. Environ. Microbiol.* 62: 80-86 (1996)), and CryIIA (Adang *et al. Plant Mol. Biol.* 21: 1131-1145 (1993)).

Pesticidal proteins produced during vegetative growth of *Bacillus* strains (vegetative insecticidal proteins, VIPs) can also be used in combination with peroxidases. For examples of VIPs see, Warren *et al.*, WO 94/21795; Warren *et al.*, WO 96/10083; and Estruch *et al.*, *Proc. Natl. Acad. Sci. USA* 93: 5389-5394 (1996).

Examples of other proteins with insecticidal compounds which can be used in combination with peroxidases include, but are not restricted to, cholesterol oxidases (US Patent 5,518,908), protease inhibitors, lectins, and  $\alpha$ -amylases.

Monocots expressing more than one insect resistance coding sequence can be made by any method known in the art. For example, the peroxidase coding sequence can be used to transform a monocot at the same time as another insect principle gene (cotransformation), the second insect principle gene can be introduced into a plant which has already been transformed with a peroxidase coding sequence, or by vice versa, or alternatively, transgenic plants, one expressing a peroxidase coding sequence and one expressing a second insect principle can be crossed to bring the coding sequences together in the same plant.

The present invention is described in further detail in the following non-limiting examples. In the Examples, procedures for making, manipulating and analyzing nucleic acids are carried out by standard procedures as described in Sambrook *et al. Molecular Cloning-A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, NY, USA (1988).

## **EXAMPLES**

### **Example 1: *Transgenic Maize Plants Comprising a Peroxidase Coding Sequence***

#### *Vector Construction*

pPOD<sub>3.5</sub> (Lagrimini *et al.*, *Proc. Natl. Acad. Sci* 84: 438-442 (1987)) contains the 1256-bp tobacco anionic peroxidase cDNA which comprises the entire peroxidase coding sequence, including a 22-amino acid signal peptide which facilitates secretion into the cell wall space. pPOD<sub>3.5</sub> was digested with *Bam*HI and cloned into the *Bam*HI sites of pCIB710 (Rothstein *et al.*, *Gene* 53:153-161 (1987)). This new construct was digested with *Eco*RI and subcloned into Bluescript SK+ (Stratagene Catalogue, 1994) - the resulting construct designated as pJS20293 (Figure 1) was placed on deposit with the Agricultural Research Culture Collection (NRRL) International Depositary Authority, 1815 N. University Street, Peoria IL 61604 U.S.A. on September 27, 1996 as NRRL B-21626. pJS20293 contains the 1200 bp peroxidase cDNA clone behind the CaMV 35S promoter and the shrunken intron (Werr *et al.*, *EMBO J.* 4: 1373-1380 (1985)) followed by the CaMV 35S terminator in a BlueScript (Stratagene) plasmid (Figure 1). pJS20293 was cotransformed with pUBI/Ac (Figure 2), a plasmid containing a chimeric bar gene coding for resistance to phosphinothricin.

#### *Transformation Using Immature Zygotic Embryos*

In two separate experiments, 600 immature embryos of CG00526, a Lancaster-type inbred, were aseptically excised 12-13 days after pollination from surface-sterilized greenhouse-grown ears. Embryos 1.5 to 20 mm in size were plated with the scutellum up on callus initiation medium, 2DG4 + 5mg/l chloramben. 2DG4 medium is Duncan's (Duncan *et al.*, *Planta* 165:322-332 (1985)) "D" medium modified to contain 20 mg/L glucose.

pJS20293 DNA was precipitated onto 1 mm gold microcarrier as described by the Dupont Biolistic manual. The DNA/gold mixture was prepared so as to deliver approximately 1 mg of pJS20293 DNA per bombardment. For the immature embryo transformation, 6.34 mg of pJS20293 + 7.21 mg of pUBI/Ac were used per 50 ml of microcarrier. Both preparations were brought to 85 ml with ethanol and 10 ml of each were dried onto macrocarriers.

Four hours prior to bombardment the embryos were transferred to 12DG4 + 5mg/L

chloramben for osmotic treatment. Thirty-six embryos on a plate were bombarded using the PDS-1000He Biolistics device according to the manufacture's directions (Dupont). The embryos were arranged on the target plate around a 2 cm diameter circle at the center of the plate with the coleorhizal ends of the scutellum all oriented in the same direction. The target plates were then angled upwards at a 30° angle so that the coleorhizal ends were hit first by the particle spray. A 24x24 mm standard screen supplied by the Biolistic manufacturer was used with rupture discs of 1550 psi value for the bombardments. Three hours following bombardment embryos were returned to 2DG4 + 5 mg/L chloramben media and then cultured in the dark at 25°C.

Fourteen days after bombardment, embryogenic responses were transferred to callus maintenance media 2DM4 + 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). M4 media is the same as G4 minus casamino acids. This media contained 5 mg/L Basta® were gradually increased to 20 mg/L.

Regeneration was initiated after 12 weeks on Basta® selection. Type I callus was subcultured onto a modified Murashige and Skoog medium (MS) (Murashige and Skoog, *Physiologia Plantarum* 15:473-497 (1962)) containing 3 % sucrose, 0.25 mg/L 2,4-D, 5 mg/l benzylzminopurine and 5 mg/l Basta® and cultured under 16 hours of light (50 mE/m-2/s-1), 8 hours dark, 25°C. Two weeks later the tissue was transferred to MS medium containing 3 % sucrose and 5 mg/l Basta®. Regenerated plants were grown on MS medium modified to contain ½ the concentration of salts and 3 % sucrose in GA7 containers.

#### *Transformation Using Type I Embryogenic Callus*

For transformation of maize using Type I embryogenic callus, the callus was obtained from immature zygotic embryos using standard culture techniques. For gene delivery, approximately 300 mg of the Type I callus was prepared by either chopping with a scalpel blade or by subculturing 3-5 days prior to gene delivery. Prior to gene delivery, the prepared callus was placed onto semi-solid culture medium again containing 12% sucrose. After approximately 4 hours, the tissue was bombarded with pJS20293 using the PDS-1000/He Biolistic device from BioRad. Two mg pJS20293 was precipitated onto 1 mm gold particles using essentially the standard protocol from BioRad. Approximately 16 hours after gene delivery the callus was transferred to standard culture medium containing 2% sucrose and 1 mg/L phosphinothricin. The callus was subcultured on selection for 8 weeks, after which surviving and growing callus was transferred to standard regeneration medium for the production of plants. The regenerated plants were assayed for resistance to

European corn borer. Resistant plants were obtained.

The resulting transgenic plants were used in a conventional plant breeding scheme to produce more transgenic plants with similar insecticidal properties. The transgenic plants were also crossed with other varieties of the same plant. The transgenic plants also produced seeds which contained the chimeric peroxidase gene stably inserted in their genome.

Transgenic maize plants containing the peroxidase coding sequence were identified by Southern blot analysis. When genomic DNA from transgenic plants was digested with the *EcoRI* restriction enzyme, a band of approximately 1.3 Kb was detectable using the peroxidase gene as specific probe signaling the presence of an intact peroxidase gene.

Transgenic maize expressing the peroxidase coding sequence were also identified by northern blot analysis. A band of approximately 1.2 Kb in size was observed in the RNA blot when hybridized with a peroxidase specific probe.

#### *Insect Bioassay*

A total of 46 transgenic maize plants were originally evaluated for insecticidal activity against *Ostrinia nubilalis* (ECB). This first group of bioassays was performed by applying 10 first instar ECB larvae to a leaf cutting that had been placed into a Gelman petri dish with a moistened filter pad to prevent the leaf cutting from drying out. The larvae were allowed to feed undisturbed for two days. Two plants from event 554 were positive in the preliminary bioassay. These two plant samples showed no signs of insect feeding and the larvae were dead. The remainder of the plants in that event and other events had healthy feeding insects.

With this activity noted, more repetitions were performed in the next ECB bioassay. Four repetitions with five larvae per repetition were done. A percent mortality reading was taken after two days. Since the plants still appeared to contain insecticidal activity, it was decided to test them against other target insects.

Transgenic maize plants expressing the peroxidase enzyme were assayed for insecticidal activity by means of insect bioassays. The procedure is similar for any maize plant transformed with any insecticidal gene but is described here using as an example a peroxidase coding sequence. One to four 4 cm sections are cut from an extended leaf of a transformed maize plant. Each leaf piece is placed on a moistened filter disc in a 50 X 9 mm petri dish. Five neonate larvae of the target insect (European corn borer, fall armyworm, corn earworm, beet armyworm and black cutworm) were placed on each leaf piece. Since

each plant was sampled multiple times this makes a total of 5-20 larvae per plant. The petri dishes were incubated at 30°C and leaf feeding damage and mortality data were scored at 24, 48, and 72 hours. The toxicity data is shown in Table I.

**Table I:** Mortality values of different insects when exposed to corn leaves expressing the peroxidase enzyme.

|                                    | <u>554-1</u> | <u>554-3</u> |
|------------------------------------|--------------|--------------|
| <i>Ostrinia nubilalis</i> (ECB)    | 100%         | 100%         |
| <i>Spodoptera frugiperda</i> (FAW) | 15%          | 15%*         |
| <i>Spodoptera exigua</i> (BAW)     | 0%           | 5%           |
| <i>Heliothis zea</i> (CEW)         | 100%         | 100%         |
| <i>Agrotis ipsilon</i> (BCW)       | 0%           | 0%           |

\* These leaves had a strong antifeeding effect against fall armyworm.

#### *Transgenic Decendants*

Transgenic maize plants from event numbers 554 and 755 (which have shown to possess insecticidal properties against European corn borer under *in vitro* bioassays) were subjected to field trials. When plants in the field reached about 40 cm of extended leaf height, infestations with ECB larvae was begun. About 300 neonate larvae mixed with corn cob grits were deposited into the whorl of each plant. Infestations continued on a weekly basis for four weeks to simulate first generation corn borer (ECB1). Starting two weeks after the initial infestation, each plant was rated weekly, and a mean ECB1 damage rating was scored (see Tables II and III). As corn plants reached anthesis, 300 neonate larvae/plant were applied weekly for four weeks to simulate second generation infestation (ECB2). About 50 days after the initial ECB2 simulated infestation, stalks were split, the tunnel damage measured (see Tables II and III). The experimental conditions are further detailed by Koziel *et al.*, *Bio/Technology* 11:194-200 (1993).

| TABLE II                      |   |  |
|-------------------------------|---|--|
| Plant Crosses<br>Plant Number | ECB1 foliar<br>damage rating <sup>1</sup> | ECB2 damage<br>Tunnel length <sup>2</sup> (cm) |
| (CG00526 x 554-3) 5 self      |   |  |
| Plant #10                     | 1   | 58   |
| Plant #15                     | 1   | 15   |
| Plant #22                     | 1   | 8  |
| (CG00526 x 554-3) 6 self      |   |  |
| Plant #4                      | 1   | 0  |
| Plant #5                      | 2   | 5  |
| Plant #9                      | 1   | 7  |
| Plant #14                     | 2   | 5  |
| Plant #19                     | 1   | 4  |
| Plant #20                     | 1   | 3  |
| Plant #25                     | 1   | 0  |
| Plant #28                     | 1   | 9  |
| Plant #29                     | 1   | 7  |
| Plant #32                     | 1   | 0  |
| Number of positives evaluated |   | 13   |
| Total tunneling               |   | 121  |
| Mean                          |   | 9.3  |
| Standard Deviation            |   | 15   |
| (Non-transformed CG00526)     |   |  |
| 1                             |   | Dead Plant, Killed by ECB                      |
| 2                             |   | Dead Plant, Killed by ECB                      |
| 3                             |   | Dead Plant, Killed by ECB                      |
| No. of negatives evaluated    |   | 3  |
| Total tunneling               |   | -  |
| Mean                          |   | -  |
| Standard Deviation            |   | -  |

<sup>1</sup> Foliar Damage ratings were determined as follows:

1. No visible leaf injury.
2. Evidence of fine "window pane" damage only on the unfurled leaf where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
3. Evidence of fine "window pane" damage on two unfurled leaves where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
4. Evidence of pin hole or shot hole feeding damage that penetrated the leaf on two or more leaves that emerged from the whorl. (any lesion <0.25" in length).
5. Elongated lesions and/or mid rib feeding evident on more than 3 leaves that emerged from the whorl. Lesions <1.0" in length.
6. Several leaves with elongated lesions (0.75" to 1.5" in length) and/or no more than one leaf with broken mid rib.
7. Long lesions (>1.0") common on about one-half of leaves and/or 2 or 3 leaves with broken mid ribs.
8. Long lesions (>1.0") common on about two thirds of leaves and/or more than 3 leaves with broken mid ribs.
9. Most leaves with long lesions. Several leaves with broken mid ribs. Possibly stunted plants due to ECB feeding.

<sup>2</sup> The extent of internal ECB tunneling damage in a 92 cm section of stalk, 46 cm above and below the primary ear node was measured on transgenic and control plants. The maximum damage that can be rated is 92 cm. Control plants were completely destroyed by the end of the experiment therefore no measurement was possible.

| TABLE III                             |   |  |
|---------------------------------------|---|--|
| Plant Crosses<br>Plant Number         | ECB1 foliar<br>damage rating <sup>1</sup> | ECB2 damage<br>Tunnel length <sup>2</sup> (cm) |
| (CG00714 x 755-9) Positive Segregants |   |  |
| Plant #6                              | 3   | 15   |
| Plant #12                             | 3   | 31   |
| Plant #16                             | 3   | 19   |
| Plant #20                             | 4   | 24   |
| Plant #24                             | 3   | 18   |
| Plant #25                             | 1   | 26   |
| (CG00714 x 755-9) Negative Segregants |   |  |
| Plant #7                              | 7   | dead plant                                     |
| Plant #8                              | dead plant                                | dead plant                                     |
| Plant #9                              | 7   | dead plant                                     |
| Plant #10                             | 7   | dead plant                                     |
| Plant #11                             | 9   | dead plant                                     |
| Number of positives evaluated         |   | 6  |
| Total tunneling                       |   | 133  |
| Mean                                  |   | 22.17  |
| Standard Deviation                    |   | 5.91   |
| No. of negatives evaluated            |   | 5  |
| Total tunneling                       |   | -  |
| Mean                                  |   | -  |
| Standard Deviation                    |   | -  |

<sup>1</sup> Foliar Damage ratings were determined as described for Table II:

1. No visible leaf injury.
2. Evidence of fine "window pane" damage only on the unfurled leaf where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
3. Evidence of fine "window pane" damage on two unfurled leaves where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
4. Evidence of pin hole or shot hole feeding damage that penetrated the leaf on two or more leaves that emerged from the whorl. (any lesion <0.25" in length).

5. Elongated lesions and/or mid rib feeding evident on more than 3 leaves that emerged from the whorl. Lesions <1.0" in length.
6. Several leaves with elongated lesions (0.75" to 1.5" in length) and/or no more than one leaf with broken mid rib.
7. Long lesions (>1.0") common on about one-half of leaves and/or 2 or 3 leaves with broken mid ribs.
8. Long lesions (>1.0") common on about two thirds of leaves and/or more than 3 leaves with broken mid ribs.
9. Most leaves with long lesions. Several leaves with broken mid ribs. Possibly stunted plants due to ECB feeding.

<sup>2</sup> The extent of internal ECB tunneling damage in a 92 cm section of stalk, 46 cm above and below the primary ear node was measured on transgenic and control plants. The maximum damage that can be rated is 92 cm. Control plants were completely destroyed by the end of the experiment therefore no measurement was possible.

**Example 2: *Transgenic Wheat Plants Comprising a Peroxidase Coding Sequence***

pJS20293 (Figure 1) and pUBIAc (Figure 2) are used to transform wheat using the methods of Chang *et al.*, WO 94/13822, Weeks *et al.*, *Plant Physiol.* 102:1077-1084 (1993) or Nehra *et al.*, *The Plant Journal* 5(2):285-297 (1994).

Transformation of wheat using one method of Chang *et al.*, WO 94/13822 is briefly set forth as follows (others methods set forth in Chang *et al.* can also be used):

***Preparation of Wheat Callus, Genotype UC703***

Wheat plants of genotype UC703 are grown to flowering and self-pollinated. Spikes containing embryos 1 to 2.5 mm in length are removed from the plants and sterilized with 10% Clorox solution for 10 minutes. Embryos are removed from the immature seeds and placed with the embryo axis downwards on the medium on Murashige and Skoog containing 5 or 10 mg/l 2,4-D, 13.7% w/v maltose, 100 mg/l proline and 100 mg/l myo-inositol solidified with 0.7-0.8% v/v phytagar or 0.1-0.2% gelrite (initiation medium). After a three week culture in the dark at 27°C, a preferred callus is recognized by the presence of

well formed globular, somatic embryos (Type M callus) developing on the scutellum of certain explants. These calli are removed and placed either on MS medium containing 1.0 to 5.0 mg/l 2,4-D and 2-3% sucrose or on a medium containing a reduced level (5%) of maltose before being placed on the sucrose medium. The material is then subcultured every week to fresh MS medium containing 3% sucrose.

#### *Cell Preparation for Bombardment*

The cells for bombardment are given a plasmolysis treatment before and after bombardment. Packed cell volume is measured and cells are diluted in 1 MS liquid medium with added osmoticum: 0.4 M sorbitol for suspension cells and 0.6 M sorbitol for callus cells. Cells are diluted such that the final packed cell volume per target is 1/30 ml for a fine suspension and 1/10 ml for callus. Diluted cells are placed in a 250 ml flask containing a stir bar and stirred for a minimum of 30 minutes, up to few hours. To plate the cells, 2 ml are withdrawn from the flask and pipetted into the top of a vacuum flask onto which a Whatman 2.5 cm GFA filter is placed. The vacuum is applied until the cells are dried onto the filter. The filters are placed on 60x15 mm petri plates containing 5 ml of solid post-bombardment plasmolysis medium, which is 1 MS containing 0.2 M sorbitol for suspension cells or 0.4 M sorbitol for callus cells. Two filters are plated on each dish.

#### *Vectors Used for Bombardment*

The following vectors into which an expression cassette containing peroxidase is inserted can be used for particle bombardment (in addition to the cotransformation of pJS20293 (Figure 1) and pUBIAC (Figure 2) using equal amounts of DNA):

pSOG30 is a b-glucuronidase (Gus) expression vector derived from plasmid pBI121, purchased from Clontech Laboratories, Palo Alto, California. Intron 6 of the maize Adh 1 gene is amplified by PCR from plasmid pB428, described in Bennetzen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:4125-4128 (1987) and ligated into the BamHI site of pBI121, which is between CaMV 35S promoter and the Gus gene. A 17 bp maize chlorotic mottle virus (MCMV) leader, described in Lommel *et al.*, *Virology* 181:382-385 (1991), is inserted into the 35S-Gus gene non-translated leader. The final gene fusion contains the structure: 35S promoter-Adh1 intron 6-MCMV leader-Gus-Nos terminator, all in the pUC19 vector backbone.

pSOG35 is a dihydrofolate reductase (dhfr) expression vector. This construct is derived by fusing the 35S promoter, Adh 1 intron 6, and MCMV leader described above to the dhfr

gene from plasmid pHCO, described in Bourouis and Jarry, *EMBO J.* 2:1099-1104 (1983). The final gene fusion contains the structure: 35S promoter-Adh 1 intron 6-MCMV leader-dhfr-Nos terminator, all in the pUC19 vector backbone.

pTG48 comprises the Gus gene under control of the anther specific ant43D promoter and a dhfr gene in a pUC19 backbone. It is the result from the combination of 4 different DNA fragments. Fragment 1 is obtained from pSOG35 after restriction cutting with HindIII and EcoRI. The EcoRI end of the isolated fragment containing the dhfr gene is adapted to a Sall restriction end. Fragment 2 consisted of the anther specific ant43D promoter isolated from plasmid pCIB 3178 after restriction cutting with HindIII and XbaI. Plasmid pCIB 3178 is described in detail in EP-578 611, the relevant parts of which are incorporated herein by reference and was deposited under accession no. NRRL B-18978. Fragment 3 is obtained from plasmid pSOG30 after restriction cutting with XbaI and EcoRI and contained the Gus gene, and fragment 4 corresponded to the commercially available vector pUC19 cut with Sall and EcoRI.

#### *Particle Preparation*

Gold particles (1.0 micron; from Bio-Rad) are washed by aliquoting into a microfuge tube, adding ~1 ml 100% ethanol, vortexing, spinning down, removing the supernatant, and repeating twice the sterile water. After the final wash, as much water is removed as possible and polylysine solution (0.02% polylysine + 15 mM ammonium acetate) is added to completely immerse the particles. The particles are vortexed, spun, and the supernatant removed. The particles are allowed to dry overnight in a laminar flow hood or for 30 minutes under a gentle nitrogen stream.

For a "full" particle preparation 10 mg particles are weighed out and placed in a sterile microfuge tube containing a stir bar. 100 ml (1 mg/ml) of each DNA (alternatively, 50 ml (1 mg/ml) of each DNA) is added, followed by vortexing. Then, 10 ml 100 mM  $\text{Na}_2\text{HPO}_4$  is added, followed by vortexing. 10 ml 100 mM  $\text{CaCl}_2$  is added, followed by vortexing. Finally, 380 ml 100% ethanol is added, followed by vortexing. While the suspension is stirred vigorously, 3 ml are pipetted onto plastic fliers (projectiles). The particles are allowed to dry onto fliers for at least 15 minutes before bombarding.

#### *Bombarding Cell Cultures*

The petri plate containing the cell filters is inverted onto the platform on top of the stage, and centered over the particle flight opening. The clear lid is placed over the top of the

platform. A microprojectile is placed onto the breech pin and the breech closed. The "arm" button is pushed to fill the reservoir with the appropriate amount of helium gas (usually 1800-1900 psi). The vacuum on the chamber is pulled to ~27 mm. After the vacuum is turned off, the "arm" and "fire" buttons are pushed. The "arm" button is then pushed to the "off" position. Each filter is usually shot twice.

#### *Post-Bombardment Culture and Selection*

After bombardment the cells are kept in the dark overnight. The next day, filters are removed from plasmolysis medium and placed on 1 MS medium. Selection is applied 1-10 days post-bombardment for suspension cells and after 14 days for callus cells. Cells are scraped off the filters and spread onto the surface of plates containing 1 MS plus 2 mg/liter methotrexate (or appropriate selective agent). Plates are incubated in the dark for several weeks. Resistant colonies that arise after a few weeks are transferred to 1MS + 4 mg/l methotrexate (or appropriate selective agent). Colonies that continue to proliferate for about 3-4 weeks are then transferred to "0.5 MS" maintenance medium, which is an aqueous solution of MS salts, vitamins, iron, 3% sucrose, 0.7% agar, 0.5 mg/liter 2,4-D. Tissue is subcultured onto this medium biweekly until embryogenic structures appeared or tissue seemed suitable for regeneration.

#### *Regeneration*

Tissue is transferred to MS medium containing either 3 mg/liter BAP or 1 mg/liter NAA + 5 mg/liter GA, and plates are moved to the light. After 2-4 weeks, tissue is transferred to MS medium without hormones. Shoots that appeared are placed in containers with either MS medium without hormones or MS medium with 0.5 mg/liter NAA. When sufficient root and shoot growth occurs, plantlets are transferred to soil and placed in a phytotron.

#### *Weeks et al., Plant Physiol. 102:1077-1084 (1993)*

Transformation of wheat using the method of Weeks *et al.*, *Plant Physiol.* 102:1077-1084 (1993) is briefly set forth as follows: wheat plants (*Triticum aestivum* L.) are grown and immature embryos 0.5 to 1 mm in length are excised from greenhouse grown plants (10-18 d after anthesis, depending on the time of year) and placed, scutellum side exposed, on callus maintenance media containing 1.5 mg/L of 2,4-D. Five days after initiation into tissue culture, proliferating callus tissue is visible at the edges of the embryos. At this stage, the embryos are bombarded with gold particles coated with 7 mg of pJS20293 and 7 mg of pUBIAc.

### *Particle Bombardment*

Prior to bombardment, 1mm gold particles are coated with pJS20293 and pUBIAc DNA by the procedure of Daines, *Biolistic Systems Newsletter* 1:1-4 (1990). A stock suspension of gold particles (Bio-Rad) are suspended at 60 mg/ml in absolute ethanol. Thirty-five microliters of the suspension are aliquoted into 1.5 ml microcentrifuge tubes, washed in sterile distilled water, and resuspended in 25 ml of Tris-EDTA containing 25 mg of supercoiled plasmid DNA. The following solutions are added in order: 220 ml of sterile water, 250 ml of 2.5 M  $\text{CaCl}_2$ , and 50 ml of 0.1 M spermidine (free base). The microfuge tubes are shaken with a vortex mixer at 4°C for 10 min and centrifuged at 16,000g for 5 min. The supernatant is removed and the pellet is washed with 600 ml of ethanol. The DNA coated gold pellets are resuspended in 36 ml of ethanol. For bombardment, 10 ml of the DNA-gold suspension is placed in the center of a macroprojectile (aka. carrier sheet).

Approximately 25 embryos are placed in the center of a 15 x 100 mm Petri dish containing callus maintenance medium solidified with 0.35% Phytigel. After 5 d in culture, the embryo-derived calli are bombarded under vacuum with pJS20293-coated gold particles, using the helium-driven DuPont Biolistic Delivery System and disposable components supplied by Bio-Rad. The distance from the stopping plate to the target is 13 cm, and the rupture disc strength is 1100 p.s.i. Immediately after bombardment, calli are transferred to MS selection media containing the appropriate amount of selective agent as can be determined by one skilled in the art.

### *Regeneration of Wheat Plants*

For regeneration, embryogenic calli are transferred to MS medium containing 0.5 mg/l dicamba as described by Hunsinger and Schauz, *Plant Breeding* 98:119-123 (1987). Calli-derived shoots are transferred to Pyrex culture test tubes containing rooting media composed of half-strength MS without hormones. For selection after bombardment, agar media at each stage is supplemented with the appropriate amount of selective agent as can be determined by one skilled in the art.

Plantlets are transferred from rooting media to pots of soil mixture and acclimated to lower humidity at 21°C in an environmental chamber. After 2 weeks, plants are transferred to the green house. These primary transgenic regenerants are called T<sub>0</sub> plants.

### *Analysis of Transgenic Plants*

Transgenic tissues and plants are analyzed using Southern and northern techniques to demonstrate the presence of the peroxidase coding sequence and RNA, respectively.

Wheat plants demonstrated to contain the peroxidase coding sequence by Southern analysis are evaluated for insecticidal activity against *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, pale western cutworm; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella fusca*, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; or *Aceria tulipae*, wheat curl mite using techniques well known in the art. Those transgenic wheat plants having insecticidal properties are subjected to field trials.

### **Example 3: *Transgenic Sorghum Plants Comprising a Peroxidase Coding Sequence***

pJS20293 (Figure 1) and pUBIAc (Figure 2) are used to transform sorghum using the method of Casas *et al.*, *Proc. Natl. Acad. Sci. USA* 90:11212-11216 (December 1993) as is briefly described as follows.

#### *Microprojectile Bombardment*

Experiments are conducted with the Biolistics PDS 1000/He system (Sanford *et al.*, *Technique J. Methods Cell Mol. Biol.* 3:3-16 (1991) using tungsten (M-25, 1.7 mm in diameter, DuPont no. 75056) or gold (1.5 - 3.0 mm in diameter, Aldrich no 32,658-5) microprojectiles. Gold (3 mg) or tungsten (0.75 mg) particles previously washed in ethanol in aqueous suspension (50 ml) are coated with 5-10 mg of plasmid DNA, as described by the manufacturer (Bio-Rad). Bombardment pressures and distances from the launching plate are experimentally determined.

Immature zygotic embryos, 10-15 per plastic Petri dish (15 x 60 mm) are bombarded

between 24 and 72 hr after culture onto medium. The embryos are transferred onto filter papers (4.5 cm in diameter) that are premoistened but not saturated with liquid medium. The filter papers function to absorb the water from the surface of the embryos, and the embryos are left for 2-3 hrs on the papers prior to bombardment. Immediately after bombardment, the immature embryos are removed from the papers and transferred to semisolid medium.

#### *Transgenic Plant Regeneration and Evaluation for Insect Resistance*

Procedures for selection and maintenance of embryogenic tissue and shoot and root formation from organized structures is as described (Cai & Butler, *Plant Cell Tissue Organ Cult.*, 20:101-110 (1990). The appropriate selective agent as known by one skilled in the art is used.

Transgenic tissues and plants are analyzed using Southern and northern techniques to demonstrate the presence of the peroxidase coding sequence and RNA, respectively.

PAT activity is evaluated in callus and leaf extracts according to DeBlock *et al.*, *EMBO J.* 6: 2513-2518 (1987).

Sorghum plants demonstrated to contain the peroxidase coding sequence by Southern analysis are evaluated for insecticidal activity against *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranean* granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes*, *Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; or *Tetranyches urticae*, twospotted spider mite using techniques well known in the art. Those transgenic sorghum plants having insecticidal properties are subjected to field trials.

#### Example 4: *Transgenic Rice Plants Comprising a Peroxidase Coding Sequence*

pJS20293 (Figure 1) and pUBIAc (Figure 2) are used to transform rice using the methods of Shimamoto *et al.*, *Nature* 338: 274-277 (1989) (rice); Christou *et al.*,

*Biotechnology* 9: 957-962 (1991) (rice); Datta *et al.*, *Bio/Technology* 8:736-740 (1990)(rice) and/or Hayashimoto *et al.*, *Plant Physiol.* 93:857-863 (1990)(rice).

Transformation of rice using the method of Christou *et al.*, *Biotechnology* 9: 957-962 (1991) is briefly set forth as follows:

#### *DNA Preparation*

DNA-coated gold particles are prepared by mixing gold particles (10 mg) with a solution of the DNA (20 mg) in 100 ml of buffer (150 mM sodium chloride, 10 mM Tris-HCl, pH 8.0) and vortexed gently for 5-10 seconds. Spermidine (100 ml of 0.1 M solution) and 100 ml of a 25% PEG solution (MW 1300-1600) are added with vortexing, followed by the dropwise addition of 100 ml calcium chloride (2.5M). The mixture is allowed to stand at room temperature for 10 minutes and then spun in a microfuge. The supernatant is removed and the precipitated gold with the DNA complex resuspended in 10 ml of 100% ethanol. The resulting suspension is then coated onto an 18 x 18 mm carrier sheet at a rate of 163 ml per carrier sheet, or a calculated rate of 0.05 mg/cm<sup>2</sup>.

#### *Isolation of Immature Embryos and Preparation for Particle Bombardment*

Twelve to fifteen-day old rice immature embryos are harvested from expanded panicles and sterilized with 2% sodium hypochlorite for five minutes. They are subsequently rinsed repeatedly with sterile distilled water and the glumes are removed under a dissecting microscope. The embryos are then aseptically removed and plated on a water-agar plate with the adaxial side in contact with the medium.

#### *Particle bombardment*

The carrier sheet bearing the beads is loaded onto the particle accelerator, which uses the discharge of a high voltage capacitor through a small water droplet as the motive force. A 100 mesh retaining screen is placed between the sheet and the target tissue suspended above the machine. The assembly is then evacuated to 500 mm Hg to reduce aerodynamic drag. Ten to sixteen Kv from a 2 mF capacitor is discharged through a 10 ml water droplet inside the expansion chamber. The sheet is thus blown against the retaining screen permitting the gold particles to continue onward to impact the target tissue suspended above the screen. The targeted immature embryos are positioned on a water-agar plate so that, when the plate is inserted over the screen, the scutellar region of the embryos would

be in the direct path of the accelerated particles.

#### *Plant regeneration*

Following particle bombardment, embryos are plated on MS or CC media supplemented with 2.4D at 0.5 or 2 mg/l and embryogenic callus and plantlets are recovered as described (Hartke, S. and Lara, H., *Genet. & Breed.* 43:205-214 (1989); Datta, S.K. *et al.*, *Plant Sci.* 67:85-88 (1990)).

#### *Recovery of Transformed Embryogenic Callus and Plants*

Transformed callus and plants are recovered under both selective and non-selective conditions. In experiments in which selection is incorporated in the transformation/regeneration protocol, the appropriate amount of selective agent as is known by those skilled in the art is used. Putative transformants thus identified, are subjected to molecular and genetic analysis to confirm stable integration and inheritance of the introduced gene.

#### *Analysis of Transgenic Plants*

Transgenic tissues and plants are analyzed using Southern and northern techniques to demonstrate the presence of the peroxidase coding sequence and RNA, respectively.

PAT activity is evaluated in callus and leaf extracts according to DeBlock *et al.*, *EMBO J.* 6: 2513-2518 (1987).

Rice plants demonstrated to contain the peroxidase coding sequence by Southern analysis are evaluated for insecticidal activity against *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; or *Acrosternum hilare*, green stink bug using methods well known in the art.

#### **Example 5: *Transgenic Oat Plants Comprising a Peroxidase Coding Sequence***

pJS20293 (Figure 1) and pUBIAc (Figure 2) are used to transform oats using the methods of Somers *et al.*, *Bio/Technology* 10:1589-1594 (Dec. 1992)(oat) and/or Torbert *et*

*al.*, *Plant Cell Reports* 14:635-640 (1995)(oat).

Transformation of oats using the method of Somers *et al.*, *Bio/Technology* 10:1589-1594 (Dec. 1992) is briefly set forth as follows:

#### *Cell Cultures*

Immature embryos of oat lines derived from GAF-30/Park are used to initiate callus (Rines, H.W. & Luke, H.H., *Theor. Appl. Genet* 71:16-21 (1985)). Friable, embryogenic callus lines are visually selected (Bregitzer, P., *et al.*, *Crop Sci* 29:798-803 (1989)) and subcultured every 2 weeks onto 0.2% Gelrite-solidified MS2D medium containing MS salts (Murashige, T. & Skoog, F., *Physiol. Plant* 15:473-497 (1962)) with 150 mg/l asparagine, 0.5 mg/l thiamine-HCl, 20 g/l sucrose and 2.0 mg/l 2,4-D, pH 5.8. Suspension cultures are initiated by placing approximately 1 g friable, embryogenic callus in 35 ml of liquid MS2D medium. Suspension cultures are selected for the presence of small aggregates of yellow-colored, cytoplasmically-dense cells and subcultured every week.

#### *Preparation of DNA-Coated Particles*

Tungsten particles are coated with pJS20293 (Figure 1) and pUBIAc (Figure 2) DNA using procedures similar to that described by Gordon-Kamm, *et al.*, *Plant Cell* 2:603-618 (1990)). Prewashed tungsten particles (1.25 mg) are resuspended in 250 ml sterile water in a 1.5 ml Eppendorf tube. A 25 ml aliquot of 1 mg/ml of each DNA, 250 ml of 2.5 M CaCl<sub>2</sub> and 50 ml of 0.1 M spermidine (free base) are added to the Eppendorf tube in that order. The mixture is vortexed using a Vortex Genie 2 (Scientific Industries, Inc.) at top speed for 1 min, placed on ice for 5 to 10 min and centrifuged at 14,000 rpm for 1 min in an 5415 Eppendorf centrifuge. Following centrifugation, 550 ml of supernatant is pipetted out and discarded. The DNA-coated tungsten particles are resuspended by pipeting them up and down several times and 1 ml of the DNA-particle suspension is loaded onto the macrocarrier of the particle delivery system.

#### *Delivery of DNA*

Suspension culture cells are rinsed 3X with MS2D medium lacking asparagine prior to bombardment. On 3 to 5 days after subculture, suspension culture cells are collected by vacuum filtration onto a 4.7 cm diameter Millipore AP 10, MF support pad (Millipore Corp.) to form an evenly spread thin layer of approximately 0.5 gm fresh weight tissue culture cells.

Pads supporting cells are then transferred to 60 x 20 mm petri dishes. For bombardment of callus, 2-week old friable, embryogenic callus (0.5 gm) is spread evenly over the Millipore support pads premoistened with 2 ml of MS2D medium minus asparagine in 60 x 20 mm Petri dishes. Petri plates containing suspension or callus samples are positioned (Gordon-Kamm, *et al.*, *Plant Cell* 2:603-618(1990)) at a distance of 5 cm from the stopping plate and bombarded with the Biolistic PDS-1000 (gun powder) Particle Delivery System (DuPont Co.).

#### *Selection of Transformants*

Following bombardment, cells are washed from each Millipore support pad with 5 ml of liquid MS2D medium lacking asparagine into a 60 x 20 mm petri disk, which is then sealed with parafilm and incubated at 21 to 23°C in the dark. After 5 days of incubation in liquid medium, bombarded cells are plated in a thin layer onto 7.0 cm diameter Whatman No. 1 filter paper disks overlying Gelrite-solidified MS2D selection medium lacking asparagine and containing 3 mg/l of phosphinothricin (PPT) (Crescent Chemical Co. Inc.). Cells from one bombardment are typically distributed over two or more filter papers depending on the cell density. Filter papers with overlying cells are transferred to fresh selection medium at 2 to 3 week intervals. PPT-resistant colonies begin to appear 7 to 8 weeks post-bombardment and are subcultured directly onto fresh selection medium without filter paper every 2 to 3 weeks thereafter.

#### *Plant Regeneration*

PPT-resistant tissue cultures are placed on N + B oat plant regeneration medium (Bregitzer, P., *et al.*, *Crop Sci* 29:798-803 (1989)) (MS salts (Murashige, T. & Skoog, F., *Physiol. Plant* 15:473-497 (1962)), 2 mg/l naphthaleneacetic acid, 0.2 mg/l benzylaminopurine) containing 3 mg/l PPT. After 2 to 6 weeks, shoots are removed from the callus and transferred to MS medium without hormones but containing 3 mg/l PPT for root formation. Rooted plants are transferred to potting soil mix and grown to maturity in growth chambers.

#### *Analysis of Transgenic Plants*

Transgenic tissues and plants are analyzed using Southern and northern techniques to demonstrate the presence of the peroxidase coding sequence and RNA, respectively.

PAT activity is evaluated in callus and leaf extracts according to DeBlock *et al.*, *EMBO J.* 6: 2513-2518 (1987).

Oat plants demonstrated to contain the peroxidase coding sequence by Southern analysis are evaluated for insecticidal activity using methods well known in the art.

**Example 6: *Transgenic Barley Plants Comprising a Peroxidase Coding Sequence***

pJS20293 (Figure 1) and pUBIAc (Figure 2) are used to transform barley using the methods of Wan *et al.*, *Plant Physiol.* 104: 37-48 (1994) and/or Jahne *et al.*, *Theor. Appl. Genet.* 89:525-533 (1994).

Transformation of barley using the method of Wan *et al.*, *Plant Physiol.* 104: 37-48 (1994) is briefly set forth as follows:

***Plant Materials***

Plants of the barley (*Hordeum vulgare* L.) spring cultivar Golden Promise are grown in growth chambers under a 16-h light/8-h dark period at 12°C and 60 to 80% humidity (Hunter, C.P., *Plant Regeneration from Microspores of Barley, Hordeum vulgare*, PhD Thesis, Wye College, University of London, Ashford, Kent (1988)). Light levels at head height are approximately 350 to 400 mE. Seeds of a winter cultivar, Igri, are germinated in soil in the growth chamber under the same conditions. When about 10 cm in height, the seedlings are vernalized for 8 weeks under a 10-h light (10-15 mE)/14-h dark period at 4°C (Hunter, C.P., *Plant Regeneration from Microspores of Barley, Hordeum vulgare*, PhD Thesis, Wye College, University of London, Ashford, Kent (1988)). After vernalization, they are grown under the same regime as Golden Promise plants. All plants are fertilized with Osmocote (Sierra, 17-6-12 plus minors) at the time of planting and then biweekly with 0.02% Verdi (Peter's, 20-20-20).

***Immature Embryos and Callus Derived from Immature Embryos***

Spikes of cv Golden Promise with immature embryos about 1.5 to 2.5 mm in size are surface sterilized in 20% (v/v) bleach (5.25% sodium hypochlorite) for 5 min, rinsed briefly three times, and washed for 5 min with sterile water. Immature embryos are dissected from young caryopses and left intact or are bisected longitudinally. For induction of callus for

bombardment, embryos (intact or bisected) are placed scutellum-side down on callus induction medium, which is Murashige and Skoog medium (Murashige, T. & Skoog, F., *Physiol Plant* 15:473-497 (1962)) supplemented with 30 g/L maltose, 1.0 mg/L thiamine-HC1, 0.25 g/L *myo*-inositol, 1.0 g/L casein hydrolysate, 0.69 g/L Pro, and 2.5 mg/L dicamba, solidified by 3.5 g/L gelrite (Scott, Carson, CA) or Phytigel (Sigma). Embryos are incubated at 25°C in the dark, and embryogenic callus is selected for bombardment after 2 weeks.

#### *Anther Culture and Microspore-Derived Embryos (MDEs)*

Spikes, wrapped by the flag leaves, are harvested from cv Igri plants when the microspores are at mid-uninucleate to early binucleate stages and surface sterilized briefly with 70% ethanol. Anthers are dissected from spikelets and 60 anthers are placed in each Petri dish (35 x 10 mm) with 3 mL of 0.3 M mannitol. The Petri dishes are sealed with Parafilm and incubated at 25°C in the dark for 3 or 4 d. Anthers are subsequently transferred into Petri dishes with 3 mL of Hunter's liquid FHG medium (a modified Murashige and Skoog medium with lower  $\text{NH}_4\text{NO}_3$  and high Gln; Kasha, K., *et al.*, "Haploids in Cereal Improvement: Anther and Microspore Culture," J.P. Gustafson, ed., *Gene Manipulation in Plant Improvement II*, Plenum Press, NY (1990), pp. 213-235). without Ficoll-400 and supplemented with 1 mg/L IAA and 0.2 mg/L kinetin (designated as FHG\*) and incubated as described. MDEs are visible after approximately 2 or 3 weeks and are used for bombardment after approximately 4 weeks.

#### *Preparation of Bombardment Plates and Microprojectile Bombardment*

One day before bombardment, IEs (1.5-2.5 mm) from young caryopses of cv Golden Promise are cut in half longitudinally and placed in three different orientations (scutellum-side up, scutellum-side down, or cut-surface up) on callus induction medium in the center of Petri dishes (100 x 15 mm). For bombardment of callus, approximately 0.5 g of embryogenic callus from cultured IEs is cut into small pieces (about 2 mm) and placed in the center of a Petri dish (100 x 15 mm) containing callus induction medium. MDEs are harvested from anther culture plates using a Pasteur pipet and distributed evenly in petri dishes (100 x 15 mm) on a piece of 5-cm Whatman No. 3 filter paper supported by two 7-cm filters. Before bombardment, excess medium is removed from the filters.

Plasmid DNA is adsorbed to gold particles (1.0 mm, Dupont, Wilmington, DE) as

described previously (Daines, R.J., *Biolistic Particle Delivery Systems Newsletter* 1:1, 4 (1990)). When two plasmids are used, equal amounts (mg) of DNA from the two plasmids are mixed. All target materials are bombarded once, using DuPont PDS 1000 He Biolistic Delivery System. The target materials are positioned approximately 13 cm below the microprojectile stopping plate; 1100-p.s.i. rupture discs are used.

### *Selection Transformants*

#### *IEs and Callus*

One day after bombardment, half-embryos and callus pieces are transferred individually to callus induction medium with 5 mg/L bialaphos; the half-embryos are cultured scutellum-side down, irrespective of their orientation during bombardment. Tissue remains on the first selection plate for approximately 10 to 14 d. At transfer to the second selection plate (5 mg/L bialaphos), individual callusing embryos or callus pieces are broken using forceps into several small pieces and maintained separately. During the subsequent two to three selection passages (each approximately 10 to 20 d, at 5 mg/L bialaphos), callus pieces showing evidence of more vigorous growth are transferred earlier to new selection plates and tissue is handled in an identical manner. All callus tissue which develops originally from each piece of embryo or callus is defined as a line. Bialaphos-resistant callus lines are maintained by monthly subculture on callus induction medium with 5 mg/L bialaphos.

#### *MDEs*

Following bombardment, several drops of FHG<sup>+</sup> medium are added to the MDEs. After 2 or 3 d, embryos of >1.5 mm are transferred individually onto callus induction medium with 3 or 5 mg/L bialaphos. Smaller embryos remain on the filters and are transferred to selection medium when they are approximately 1.5 mm. Filter papers are washed every 2 or 3 d by repeated addition and removal of liquid FHG<sup>+</sup> medium. MDEs remain on the first selection medium for 10 to 20 d. MDEs showing evidence of callus formation are transferred to fresh selection medium with 5 mg/L bialaphos. During transfer, each callusing MDE is broken into a few small pieces. Further selection is as described in the previous section.

### *Plant Regeneration and Herbicide Application*

Plants are regenerated from PAT-positive callus lines by transferring embryogenic callus to FHG medium with 1 mg/L bialaphos at 23° or 25°C under fluorescent lights (45-55 mE, 16 h/d). In approximately 2 weeks, plantlets are observed. Green plantlets, approximately 2

cm, are transferred into Magenta boxes containing plantlet growth medium (hormone-free callus induction medium) with 1 mg/L bialaphos. Before they grow to the top of the box, plantlets are transferred to 6-inch pots containing Supersoil and placed in the greenhouse (16-h light period, 15°-18°C). Regenerants grow to maturity and are self-pollinated. Some of the plants are tested for their response to Basta (200 g/L PPT, Hoechst AG, Frankfurt, Germany) by spraying with a 0.5% (v/v) solution plus 0.1% Tween 20. Plants are also regenerated from wild-type callus on the media without bialaphos.

#### *Analysis of Transgenic Plants*

Transgenic tissues and plants are analyzed using Southern and northern techniques to demonstrate the presence of the peroxidase coding sequence and RNA, respectively.

Barley plants demonstrated to contain the peroxidase coding sequence by Southern analysis are evaluated for insecticidal activity against *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Hylemya platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Thysanoptera*, Thrips; or *Petrobia latens*, brown wheat mite using methods well known in the art.

\* \* \* \* \*

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

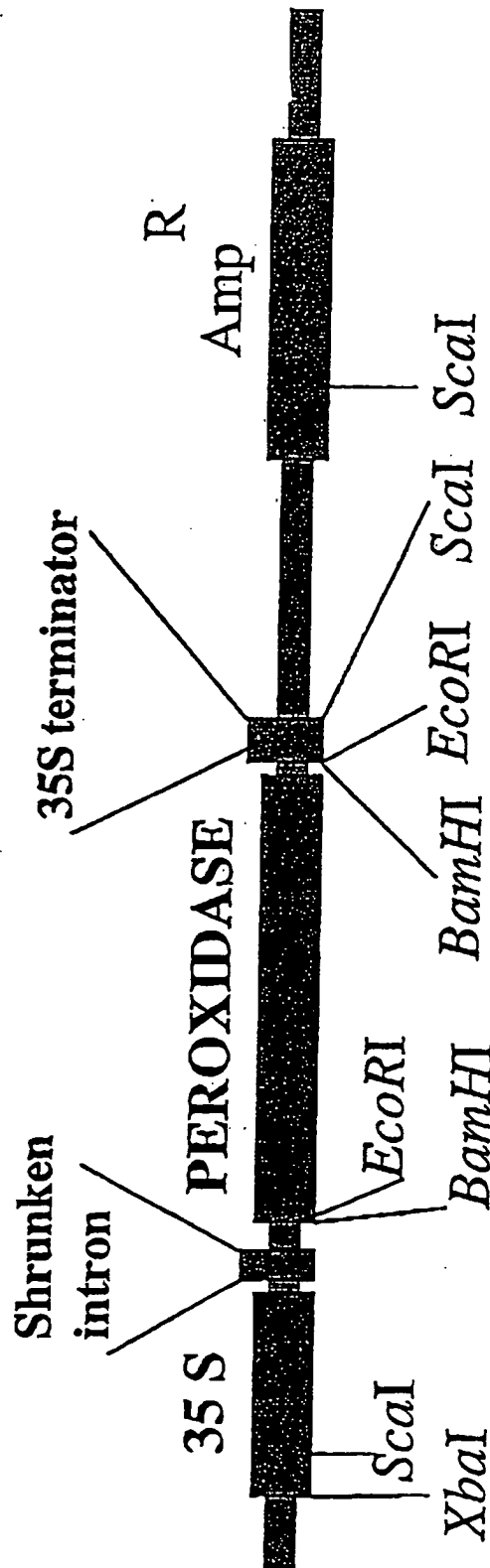
***What is claimed is:***

1. A method for controlling insects comprising feeding or contacting an insect with an insecticidal amount of transgenic monocotyledonous plant cells, wherein said plant cells comprise recombinant DNA comprising a coding sequence encoding peroxidase.
2. The method according to claim 1, wherein said method comprises transforming a monocotyledonous plant with a DNA sequence comprising a coding sequence encoding peroxidase.
3. The method of claim 1, wherein said DNA sequence comprises a promoter operably linked to said coding sequence.
4. The method of claim 3, wherein said DNA sequence comprises a chimeric gene comprising a promoter operably linked to said coding sequence.
5. The method of claim 1, wherein said peroxidase is an anionic peroxidase.
6. The method of claim 1, wherein said cells comprise a part of a fertile transgenic plant.
7. The method of claim 6, wherein said plant is selected from the group consisting of wheat, rice, oat, barley, sorghum and maize.
8. The method of claim 7, wherein said plant is a maize plant.
9. The method of claim 8, wherein said maize plant is from a maize line selected from the group consisting of CG00526, CG00615, and CG00714.
10. The method of claim 1, wherein said plant is transformed using a technique selected from the group consisting of particle bombardment, electroporation, and polyethylene glycol treatment.
11. The method of claim 1 wherein said DNA sequence further comprises a selectable or screenable marker gene.

12. The method of claim 11, wherein said marker gene encodes an enzyme selected from the group consisting of neomycin phosphotransferase, hygromycin phosphotransferase, dihydrofolate reductase, phosphinothricin acetyltransferase, 2, 2-dichloropropionic acid dehalogenase, acetohydroxyacid synthase, 5-enolpyruvyl-shikimate-phosphate synthase, haloarylnitrilase, acetyl-coenzyme A carboxylase, dihydropteroate synthase, chloramphenicol acetyl transferase, and  $\beta$ -glucuronidase.
13. The method of claim 1, wherein said insect is from an order selected from the group consisting of Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, and Trichoptera.
14. The method of claim 13, wherein said insect is selected from the group consisting of European corn borer, stalk corn borer, black cutworm, corn earworm, fall armyworm, southwestern corn borer, lesser cornstalk borer, sugarcane borer, western corn rootworm, northern corn rootworm, southern corn rootworm, wireworms, northern masked chafer, southern masked chafer, Japanese beetle, corn flea beetle, maize billbug, corn leaf aphid, corn root aphid, chinch bug, redlegged grasshopper, migratory grasshopper, seedcorn maggot, corn blotch leafminer, grass thrips, thief ant and two-spotted spider mite.
15. A fertile transgenic monocotyledonous plant comprising recombinant DNA comprising a coding sequence encoding peroxidase, wherein expression of peroxidase confers on said monocotyledonous plant a phenotypic trait.
16. The plant of claim 15, wherein said phenotypic trait is selected from the group consisting of insect resistance and standability.
17. The plant of claim 16, wherein said phenotypic trait is insect resistance.
18. The plant of claim 15, wherein said peroxidase coding sequence is an anionic peroxidase gene.

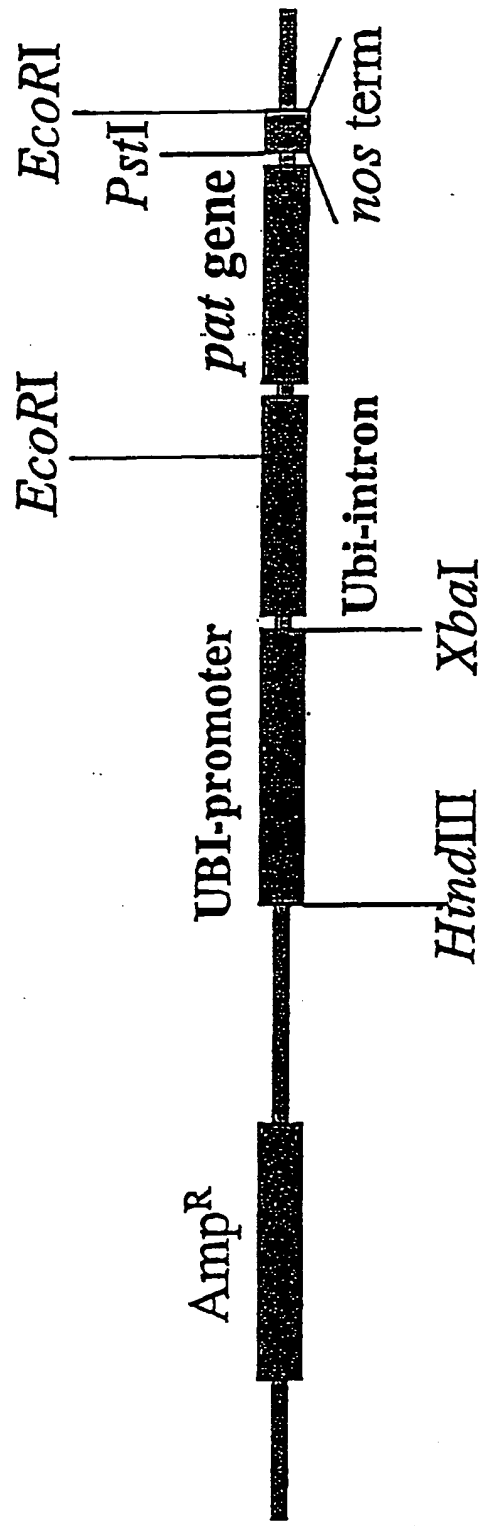
19. The plant of claim 15, wherein said plant is selected from the group consisting of wheat, rice, oat, barley, sorghum and maize.
20. The plant of claim 19, wherein said plant is a maize plant.
21. The plant of claim 20, wherein said maize plant is from a maize line selected from the group consisting of CG00526, CG00615, and CG00714.
22. The plant of claim 15, wherein said DNA sequence further comprises a selectable or screenable marker gene.
23. The plant of claim 22, wherein said marker gene encodes an enzyme selected from the group consisting of neomycin phosphotransferase, hygromycin phosphotransferase, dihydrofolate reductase, phosphinothricin acetyltransferase, 2, 2-dichloropropionic acid dehalogenase, acetohydroxyacid synthase, 5-enolpyruvyl-shikimate-phosphate synthase, haloarylnitrilase, acetyl-coenzyme A carboxylase, dihydropteroate synthase, chloramphenicol acetyl transferase, and  $\beta$ -glucuronidase.
24. A transgenic plant cell, tissue, or seed obtained from the plant according to claim 15.
25. A transgenic descendant of the plant according to claim 15.
26. A transgenic plant cell, tissue, or seed obtained from the descendant according to claim 25.
27. Use of a recombinant DNA encoding peroxidase to confer insect resistance to monocotyledonous plant cells.
28. Use of a plant comprising recombinant DNA encoding peroxidase to control damage to the plant caused by insect attack.
29. An agricultural method wherein use is made of transgenic plant material or plants comprising recombinant DNA comprising a coding sequence encoding peroxidase, wherein expression of peroxidase confers on said monocotyledonous plant a phenotypic trait.

1/2



1 Kb

Fig. 1. Plasmid pJS20293.



1 Kb

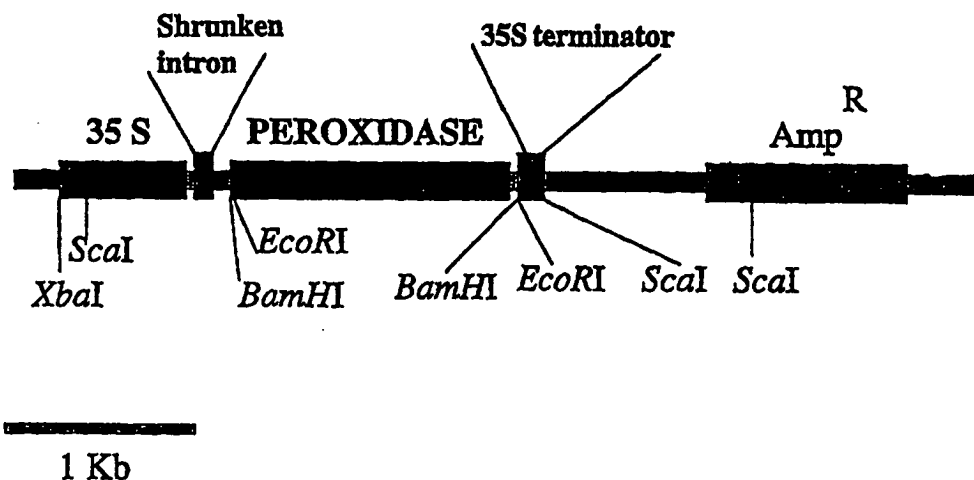
Fig. 2. Plasmid pUBIAC.



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (71) Applicants (for all designated States except US): NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH-4058 Basel (CH). OHIO STATE UNIVERSITY [US/US]; Research Foundation, 1960 Kenny Road, Columbus, OH 43210-1063 (US). U.S. DEPARTMENT OF AGRICULTURE [US/US]; Washington, DC 20250-0101 (US).  |  |  |  |
| (72) Inventors; and<br>(75) Inventors/Applicants (for US only): PRIVALLE, Laura, Stein [US/US]; 3101 Wild Meadow Drive, Durham, NC 27705 (US). LAGRIMINI, Lawrence, Mark [US/US]; 4825 Wallington Drive, Hilliard, OH 43026 (US). DOWD, Patrick, Francis [US/US]; 2311 Manito Court, Peoria, IL 61614 (US). ESTRUCH, Juan, Jose [ES/US]; 2911 E. Bainbridge Drive, Durham, NC 27713 (US). |  |  |  |
| (74) Agent: ROTH, Bernhard, M.; Novartis AG, Patent- und Markenabteilung, Lichtstrasse 35, CH-4002 Basel (CH).  |  | (88) Date of publication of the international search report:<br>30 July 1998 (30.07.98)  |  |

(54) Title: METHODS FOR CONFERRING INSECT RESISTANCE TO A MONOCOT USING A PEROXIDASE CODING SEQUENCE



## (57) Abstract

The present invention relates, in general, to methods and compositions for controlling insects in monocotyledonous plants (monocots), particularly maize. More precisely, the present invention relates to (1) a method for controlling insects comprising feeding or contacting an insect with an insecticidal amount of transgenic monocotyledonous plant cells comprising a recombinant DNA sequence comprising a coding sequence encoding peroxidase and (2) a fertile transgenic monocot plant comprising a recombinant DNA sequence comprising a coding sequence encoding peroxidase.

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# INTERNATIONAL SEARCH REPORT

National Application No  
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**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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|------------|---|-----------------------|
| X          | DENG W. ET AL.: "Plant lipid peroxidation manipulation and effects on aphid resistance"<br>PLANT PHYSIOLOGY SUPPLEMENT,<br>vol. 99, no. 1, May 1992,<br>page 109 XP002066727<br>see abstract  | 28                    |
| X          | ---<br>DOWD P. AND VEGA F.: "Enzymic oxidation products of allochemicals as a basis for resistance against insects: effects on the corn leafhopper Dalbulus maidis"<br>NATURAL TOXINS,<br>vol. 4, 1996,<br>pages 85-91, XP002066728<br>* see esp. p. 88 l. 48-51 *<br>---<br>-/-- | 28                    |

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NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

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| Category | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No.     |
|----------|---|---------------------------|
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